7th International Hydrogenase Conference

The University of Reading

August 24th to 29th

Welcome

Welcome to the 7th International Hydrogenase Conference. Every time this conference has been held we have heard about ground breaking developments in the field. This conference promises to be no different. We hope that you enjoy the conference and leave feeling inspired by what you have learned.

The local organising committee.

Dick Cammack
Richard Dinsdale
Frank Sargent
Rob Robson

7th International Hydrogenase Conference

The University of Reading
24th to 29th August, 2004

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European Office of Aerospace Research and Development.

The Biochemical Society
has also adopted this Conference as an "Independent Meeting" and we are grateful for their support

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Conference Programme.

Key to venues: C, Cedar Room, **PB**, Palmer Building Conference Centre; **WH**, Windsor Hall.

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13.00		Registrations	PB-Foyer
20.00	21.00	Opening Session. Chair: **Rob Robson** T1 **August Böck** Opening Lecture. *Molecular Biology of Formate Hydrogenlyase: Regulation of Synthesis and Maturation.*	PB-G10
21.00	22.30	Conference Reception	PB-Foyer

Wednesday 25th August.

Main Theme: Enzyme Structure, Mechanism and Chemistry Session Chair. **Fred Hagen**			
Start	**End**	**Event/Session**	**Venue**
9.00.	9.40	T2. **Juan Fontecilla-Camps.** (Keynote Talk). *Structural basis for the different activation properties of the ready and unready states of [Ni-Fe]-hydrogenases.*	PB G-10
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10.50	11.20	Coffee	PB-Foyer
11.20	11.40	T5. **Marcetta Dahrensbourg** *Asymmetric models of the active site of [FeFe]H_2ase and electrocatalysis: as good as platinum or as good as it gets.*	PB G-10
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12.20	12.40	T8. **Wolfgang Lubitz** *EPR experiments to elucidate the structure of the ready and unready states on the [Ni-Fe] hydrogenase of Desulfovibrio vulgaris miyazaki F.*	PB G-10
12.40	14.00	Lunch	C
14.00	17.00	Poster Session 1	PB-Foyer
15.30	16.00	Coffee	PB-Foyer
17.00	18.00	T9. **Rolf Thauer** (Kenote Talk) *The "metal-free" hydrogenase (Hmd) contains functional iron after all.*	PB G-10
19.00	21.00	Dinner	C

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Start	**End**	**Event/Session**	**Venue**
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11.10	11.40	T13. **Kornél Kovács** *The hydrogenases of Thiocapsa roseopersicina.*	PB G10
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15.30	16.00	Coffee	
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9.40	10.20	T24. **Jannecke Balk** *The hydrogenase-like Narlp is essential for maturation of cytosolic and nuclear iron-sulphur proteins.*	PB G10
10.20	10.20	T25. **Dennis Dean** *Biological Iron-Sulfur Cluster Formation.*	PB G10
10.40	11.10	Coffee	PB Foyer
11.10	11.30	T26. **Melanie Blokesh** *The cyanide Ligands of [NiFe] Hydrogenases in E. coli: From HypE to the HypC x HypD Complex.*	PB G10
11.30	11.50	T27. **Jose Palacios** *Maturation and biodiversity of hydrogenases from legume endosymbiotic bacteria.*	PB G10
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T29	Identification of the [Fe]-hydrogenase assembly genes, HydEF and HydG, in Chlamydomonas reinhardtii. *Matthew C. Posewitz, Paul W. King, Sharon L. Smolinski, Liping Zhang, Maria L. Ghirardi, Michael Seibert*	p49
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T1. Molecular Biology of Formate Hydrogenlyase: Regulation of Synthesis and Maturation

August Boeck

Department Biology I, University of Munich, Germany.

The disproportionation of formate into H_2 and CO_2 catalysed by formate hydrogenlyase (FHL) has attracted the attention of microbial physiologists since its discovery more than 70 years ago [1] and served to address questions of general biological relevance. Gest and Peck [2] first showed that the activities of a formate dehydrogenase, electron carriers and a hydrogenase are involved. The inactivation of the genes required for generation of FHL activity then indicated that the functions of at least seven proteins participate in this simple chemical reaction. The majority of them display sequence similarities with components of the NADH ubiquinone oxidoreductase complex, supporting the early suggestion of a primitive membrane energization function [3] now proven by the properties of other members of the Ech hydrogenase family which are proton pumping [4]. The synthesis of active FHL needs the availability of four metals (Fe, Ni, Mo and Zn) and of the half metal Se. FHL therefore, provides an excellent model system for studying the mechanisms via which complex metal centres are assembled. In this lecture, an overview will be presented on the activities of proteins involved in the insertion of iron and nickel into the large hydrogenase subunit and on the maturation of the active site. In a second topic, flux control will be discussed. FHL is synthesised during fermentative growth which is the "last resort" of energy metabolism giving the minimal yield compared to respiratory processes. Intricate regulatory circuits have evolved which control the flux through this pathway adjusting it to the availability of external electron acceptors and metals and to the needs of pH homoeostasis and oxygen stability.

[1] M. Stephenson and L. H. Stickland. Biochem. J. 26, 712-724 (1932)
{2} H. Gest and H. D. Peck J. Bacteriol. 70, 326-334 (1955)
[3] B. A. Haddock and C. W. Jones. Bact. Rev. 41, 47-99 (1977)
[4] J. Meuer, C. Kuettner, K. J. Zhang, R. Hedderich and W. W. Metcalf. Proc. Natl. Acad. Sci. USA 99, 5632- 5637 (2002)

T2. Structural basis for the different activation properties of the ready and unready oxidized states of [NiFe]-hydrogenases.

Anne Volbeda,† Lydie Martin,† Christine Cavazza,† Bart W. Faber,‡ Winfried Roseboom,‡ Simon P. J. Albracht,‡ Marc Rousset§ and Juan C. Fontecilla-Camps,†

†Laboratoire de Cristallographie et de Cristallogenèse des Protéines, Institut de Biologie Structurale J.P. Ebel (CEA-CNRS-UJF), 41 rue Jules Horowitz, 38027 Grenoble Cédex 1, France; ‡Swammerdam Institute for Life Sciences, Biochemistry, University of Amsterdam, Plantage Muidergracht 12, NL-1018 TV Amsterdam, The Netherlands; §Unité de Bioénergétique et Ingénierie des Protéines, Institut de Biologie Structurale et Microbiologie, CNRS, 31 chemin Joseph Aiguier, Marseille Cédex 20, France.

[NiFe]-hydrogenases catalyse the reversible heterolytic cleavage of molecular hydrogen. Several oxidized, inactive states of these enzymes are known that are distinguishable by their very different activation properties. So far, the structural basis for this difference has not been understood because of lack of relevant crystallographic data. Here, we present the crystal structure of the ready Ni-B state of *D. fructosovorans* [NiFe]-hydrogenase and show it to have a putative m-hydroxo Ni-Fe bridging ligand at the active site. On the other hand, a new, improved refinement procedure of the X-ray diffraction data obtained for putative unready Ni-A and Ni-SU states suggests that they may contain a bridging (hydro-) peroxide ligand. The slow activation of Ni-A, compared to the rapid activation of Ni-B, is therefore proposed to result from either the slow release of hydrogen peroxide from the active site or the additional two-electron reduction needed to convert peroxide to water or hydroxide. The possible function of Fe hydrogenase-like protein from eucaryotes will also be discussed.

T3. Hydrogenases: CO, light sensitivity and more.

Simon P.J. Albracht and Winfried Roseboom

Swammerdam Institute for Life Sciences, Biochemistry, University of Amsterdam, Plantage Muidergracht 12, NL-1018 TV Amsterdam, The Netherlands

In co-operation with E. Claude Hatchikian (Unité de Bioenergetique et Ingenierie des Proteines, Marseille, France); Erica J. Lyon, Seigo Shima and Rudolf K. Thauer (Max Planck Institute for Terrestrial Microbiology, Marburg, Germany); V. Codrina Popescue and Eckard Münck (Carnegie Mellon University, Dept. of Chemistry, Pittsburgh, Pa, USA); Melanie Blokesch, Nikola Drapal and August Böck (Ludwig-Maximilians-Universität, Dept. I der Fakulät für Biologie, München, Germany).

It is now clear that all known hydrogenases contain Fe with one or more CO ligands. In model compounds metal-carbonyl bonds are light sensitive. Some examples of light-sensitive hydrogenases will be presented. Illumination of the Nia-C* state of [NiFe]-hydrogenases at 70 K induces large changes in the nickel-EPR spectrum and the IR spectra of the CN and CO ligands to iron. This transition has been studied by Mössbauer spectroscopy. The results will be shown and the possible implications for the Nia-C* state will be discussed. The carbon source for the CN groups in hydrogenase-3 from *Escherichia coli* has been uncovered as carbamoylphosphate [1]. The origin of CO is not yet known. We succeeded to incorporate 13C into the CO group, but not into the CN groups, of the Ni-Fe site of the *Allochromatium vinosum* enzyme, using a simple 13C source in a growth medium with a 20-fold excess of unlabelled bicarbonate. Possible explanations will be discussed.

[1] Reissmann, S, Hochleitner, E, Wang, H, Paschos, A, Lottspeich, F, Glass, RS, and Böck, A (2003) Science 299: 1067-1070.

T4. Iron Carbonyl Comes to Life: Progress in Modeling the H Cluster of the Fe-only Hydrogenase

Thomas B. Rauchfuss

Department of Chemistry, University of Illinois
Urbana, Illinois 61801, U.S.A.

The unusual structural features of the Fe-only hydrogenase active site attracted much attention from chemists and biochemists. Accurate structural analogues of this site promise to inform the underlying mechanistic enzymology. Furthermore, such analogues could present new opportunities for applied catalysis.

Soon after the break-through reports from Peters, Fontecilla-Camps and their coworkers, modelling focused essentially on the chemistry of $Fe_2(SR)_2(CO)_4L_2$ where L = cyanide and PR3. Now, four years later, we review progress, focusing on challenges and opportunities for chemists and biochemists alike.

The following themes will be discussed:
1) Models for the oxidized states of the binuclear site,
2) Properties of the corresponding Ru analogues,
3) Areas of "incomplete progress" including biomimetic catalysis and the dithiolate cofactor.

T5. Asymmetric Models of the Active Site of [FeFe]H_2ase and Electrocatalysis: As Good as Platinum or as Good as It Gets?

Marcetta Y. Darensbourg, Rosario Mejia-Rodriguez, Jesse W. Tye, Jonghyuk Lee, Daesong Chong, Manuel P. Soriaga

Department of Chemistry, Texas A&M University, College Station, TX 77843, USA

Strategies for the development of synthetic analogues of hydrogenase active sites that might function as catalysts for hydrogen uptake or hydrogen production have been inspired by continuing advances in the understanding of the fundamental chemistry of dinuclear organometallic iron complexes as exist in the [FeFe]H_2ase active site. Taking nature's example of asymmetry in the active site as well as computations of model complexes which show the importance of such asymmetry to coordination sphere flexibility (see poster by Jesse Tye), we have explored electrocatalysis of H_2 production using the au courant N-heterocyclic carbene ligand, 1,3-bis(2,4,6-trimethylphenyl)-imidazol-2-ylidene, IMes, for an experimental study of a singly substituted derivative of (μ-pdt)[Fe(CO)$_3$]$_2$. NHC's are 2-dimensional ligands which offer minimal steric hindrance on their flat side, while on the other the steric dimensions possible from substituents on nitrogen are limitless. The extreme bulk of the IMes ligand locks in asymmetry into the model complex, [(μ-pdt)[Fe(CO)$_3$] [Fe(CO)$_2$IMes], or 1-IMes; its molecular structure and physical properties, will be reported. Our objective is to determine whether an asymmetric complex which has such a sterically encumbered ligand on one end might have special features conducive to H_2 electrocatalysis or H_2 uptake and activation. The electrocatalysis of H_2 production is performed on glassy carbon electrodes and compared to platinum. NHC's, whose scope as ligands in catalysis has been described as "revolutionary", are often referred to as phosphine mimics due to the similarities in synthetic methods for the formation of MLx fragments. They are actually better donors than phosphines, more closely matching cyanide according to Eisenstein, Crabtree, et al. [1]. They form strong M=C bonds and the resulting robust complexes are typically less O_2 sensitive than phosphines. Might they have a role in nature? Intriguingly, protonated NHC's share some features with the cofactor of Thauer's iron-sulfur cluster free [Fe]H_2ase.

[1] L. Perrin; E. Clot; O. Eisenstein; J. Loch; R. H. Crabtree; *Inorg. Chem.* 2001, *40*, 5806-5811.

T6. Iron-only hydrogenase: synthetic sub-sites , H-cluster assemblies and solid-state materials

Chris Pickett, Cedric Tard, Xiaoming Liu and Saad Ibrahim

Department of Biological Chemistry, John Innes Centre, Norwich NR4 7UH, U.K.

The synthesis and reactivity of structures related to the sub-site and the H-cluster catalytic unit of the iron-only hydrogenases will be described. How such assemblies can be confined in solid-state electrode materials will be shown.

T7. Modelling Metalloenzymes: Nickel-iron and Iron-only Hydrogenases

Shuqiang Niu, Lisa Thomson, Shuhua Li, Zexing Cao, Hua-Jun Fan, Yubo Fan, Alejandro Pardo, and Michael B. Hall

Department of Chemistry, Texas A&M University, College Station, Texas 77843 USA

The catalytic cycles for H_2 oxidation in [NiFe] and [Fe-only] hydrogenases have been investigated through density functional theory (DFT) for a wide variety of redox and protonated structures of the active site models, (CO)(CN)$_2$Fe (□-Sme)$_2$Ni(Sme)$_2$ and L(CO)(CN)Fe((□-SCH$_2$)$_2$X)(□-CO)Fe(CO)(CN)(SMe), respectively. By combining a calibration curve for the calculated CO bond distances and frequencies and the measured IR stretching frequencies from related complexes with the DFT calculations on the [NiFe] model, the redox states and structures of the active site are predicted. Dihydrogen activation on the Fe(II)-Ni(III) species is more favorable than on the corresponding Ni(II) or Ni(I) species. Our final proposed structures are consistent with IR, EPR, and ENDOR measurements and the correlation coefficient between the measured CO frequency in the enzyme and the CO distance/frequency calculated for the model species is excellent. The unconstrained optimized geometries for high-spin Ni(II) species and for the Ni(III) species involved in the H_2-cleavage reaction, especially the transition state, show remarkable structural resemblance to the active site in the enzyme crystal structure. For the [Fe-only] modeling, full frequency calculations on models for the active site and for well-characterized complexes show that observed and catalytically active redox species in the enzyme must correspond to Fe(II)-Fe(II), Fe(II)-Fe(I), and Fe(I)-Fe(I). Furthermore, when X is NH rather than CH$_2$, a single Fe and this N create a very favorable thermodynamic path for the heterolytic cleavage of H_2. The H_2-cleaved species shows an unusually short "dihydrogen bond", Fe—H - - - - H—N.

T8. EPR experiments to elucidate the structure of the ready and unready states of the [NiFe] hydrogenase of *Desulfovibrio vulgaris* Miyazaki F

Wolfgang Lubitz, Maurice van Gastel, Caroline Fichtner, Frank Neese

Max-Planck-Institut für Bioanorganische Chemie, Stiftstrasse 34-36, D-45470 Mülheim an der Ruhr, Germany

Isolation and purification of the [NiFe] hydrogenase of *D. vulgaris* Miyazaki F under aerobic conditions leads to a mixture of two states, Ni-A (unready) and Ni-B (ready). Ni-B can be readily activated under hydrogen whereas this process requires much longer times in case of Ni-A. The two states are distinguished by different g values in the EPR spectra. Their Ni 61 hyperfine couplings as determined from EPR on Ni 61-enriched hydrogenase are very similar, indicating no major difference of the electronic structures of the two states. Based on H ENDOR and HYSCORE data of the respective Ni-A and Ni-B samples we find that both states have an exchangeable proton, albeit with a different proton hfc tensor. This proton is assigned to the bridging ligand between Ni and Fe. Thus, it is postulated that both Ni-A and Ni-B carry a protonated oxygenic ligand, revising previous assignments. To explain the structural difference between Ni-A and Ni-B different models are presented. These include different ligands (OH vs. OOH) and also different orientations of the bridging ligand. For possible distinctions, DFT calculations are employed. A model is presented that can explain the difference in activation rates of the Ni-A and Ni-B states.

T9. The "metal-free" hydrogenase (Hmd) contains functional iron after all.

Rudolf K. Thauer

Max-Planck-Institute for Terrestrial Microbiology, Karl-von-Frisch-Strasse, D-35043 Marburg, Germany

In many methanogenic archaea, when growing under conditions of nickel limitation, there is an enzyme that catalyses the reversible reduction of methenyltetrahydromethanopterin with H_2, which is an intermediary step in the reduction of CO_2 to methane. This unusual hydrogenase (Hmd) was until recently considered to be "metal-free" since it lacks iron-sulfur clusters and nickel and its proposed catalytic mechanism does not require the presence of a redox acive transition metal. Four years ago the hydrogenase was found to contain a tightly bound organic cofactor, which is required for activity. [1] The cofactor, which is stable in the presence of 50 mM EDTA, turned out to be light sensitive. [2] Upon light inactivation 1 mol Fe, 2 mol CO and 1 mol of (6-carboxymethyl-3,5-dimethyl-2-pyridone-4-yl)-(5´-guanosyl) phosphate were generated indicating that the organic cofactor contains iron complexed by the novel pyridone, by 2 CO and by additional not yet identified ligands. [3] This was in the mean time substantiated by IR-, Mössbauer- and XA spectroscopy.

[1] Buurman, G, Shima, S, & Thauer, RK (2000) FEBS Lett. 485: 200-2004.

[2] Lyon, EJ., Shima, S, Buurman, G, Chowdhuri S, Batschauer, A, Steinbach, K, & Thauer, RK (2003) Eur. J. Biochem. 27: 195-204.

[3] Shima, S, Lyon, EJ, Sordel-Klippert, M, Kauß, M, Kahnt, J, Thauer, RK, Steinbach, K, Xie, X, Verdier, L, & Griesinger, C (2004) Angew. Chem. Int. Ed. 43:

T10. A multiprotein complex mediates H_2-dependent hydrogenase gene expression in *Ralstonia eutropha*

Oliver Lenz, Thorsten Buhrke, Antje Porthun and Baerbel Friedrich

Institut fuer Biologie/Mikrobiologie, Humboldt-Universitaet zu Berlin, Chausseestrasse 117, D-10115 Berlin, Germany

The faculatative chemolithoautotrophic proteobacterium *Ralstonia eutropha* lives in the anoxic-oxic interface, an enviroment in which H_2 occurs only temporarily in trace amounts [1]. Therefore, the costly hydrogen-oxidizing enzyme system in *R. eutropha* , consisting of two [NiFe]-hydrogenases, a the membrane-bound and an NAD-reducing soluble hydrogenase, is tightly regulated on the transcriptional level in response to the availability of hydrogen and by a global catabolite-control of yet unknown mechnism [2]. H_2-sensing in *R. eutropha* is mediated by a cytoplasmic [NiFe]-hydrogenase (RH) which communicates with a sigma^{54}-dependent HoxJ-HoxA two-component regulatory system [3]. The RH forms together with the PAS-containing histidine protein kinase HoxJ a tight complex, presumably consisting of two RH heterodimers and a HoxJ homotetramer [4]. First biochemical analysis of this 350-kDa complex revealed that HoxJ is phosphorylated in the absence of H_2 and remains unphosphorylated if H_2 becomes available. This result is in perfect agreement with the observation, that the response regulator HoxA triggers hydrogenase gene expression in its non-phosphorylated conformation [3]. This "inverse" activation mechanism of HoxA is clearly distinct from that of orthodox response regulators which are usually active in the phosphorylated form.

[1] Conrad, R (1996) Microbiol. Rev. 60:609-640.
[2] Schwartz, E, Friedrich, B (2003), Springer-Verlag, New York, http://link.springer-ny.com/link/service/books/10125/.
[3] Lenz, O, Friedrich, B (1998) Proc. Natl. Acad. Sci. USA 95:12474-12479.
[4] Buhrke, T, Lenz, O, Porthun, A, Friedrich, B (2004) Mol. Microbiol. 51:1677-89.

T11. Transcription control of hydrogenase gene expression in *Rhodobacter capsulatus*

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H_2-uptake [NiFe]hydrogenases are linked to respiratory chains; they allow energy-conserving H2 oxidation [1]. Their synthesis is regulated at the transcriptional level [2]. Transcription of the *hupSL* genes, which encode the uptake [NiFe]hydrogenase of *Rhodobacter capsulatus*, is specifically activated by H_2. Three proteins are involved: the H_2-sensor HupUV, the histidine kinase HupT and the transcriptional activator HupR. *HupT* and *hupUV* mutants have the same phenotype, i.e. an increased level of *hupSL* expression (assayed by *phupS::lacZ* fusion), even in the absence of H_2; they negatively control *hupSL* gene expression [3]. HupT is capable of autophosphorylation [4]; *in vitro* phosphotransfer to its cognate response regulator HupR was demonstrated [5]. The nonphosphorylated form of HupR binds to a palindromic sequence of *phupS* centered at -157 from the transcription start site and requires IHF to fully activate *hupSL* transcription [5, 6]. HupUV is an O_2-insensitive [NiFe]hydrogenase [7], which interacts with HupT in order to regulate the phosphorylation state of HupT in response to H_2 availability. The N-terminus PAS domain of HupT is absolutely required for the interaction with HupUV [8]. This interaction is weakened in the presence of H_2, but incubation of HupUV with H_2 has no effect on the stability or the dimer/tetramer equilibrium of HupUV. HupSL synthesis is also under the control of the global two-component regulatory system RegB/RegA [9, 10], which controls gene expression in response to redox [10]. RegA binds to a site close to the -35 promoter recognition site and to a site overlapping the IHF DNA-binding site, and acts as a repressor [9].

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T12. Molecular and functional caracterization of HupE proteins in *Rhizobium leguminosarum* bv. viciae

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The hydrogen oxidation system of *Rhizobium leguminosarum* bv viciae strain UPM791 contains a [NiFe] hydrogenase and its synthesis involves a gene cluster of 18 components (*hupSLCDEFGHIJKhypABFCDEX*) located in the symbiotic plasmid. The precise functional role of many of these genes is still unknown. Particularly intriguing is the function of *hupE* gene. Besides in R. leguminosarum, its presence has only been detected in the hup systems of *Methylococcus capsulatus*, *Rhodobacter spheroides* and *Azorhizobium caulinodans*. The molecular structure of HupE as an integral membrane protein, and its homology to UreJ, a component of the urease system from *Bordetella bronchiseptica* [1], suggest a role as a specific nickel transporter for hydrogenase.

Previously performed experiments with a *hupE* mutant of *R. leguminosarum* UPM791 revealed no significant differences on symbiotic hydrogenase activity levels as compared with the wild-type strain. However, the analysis of the genomic sequence of *R. leguminosarum* bv viciae strain 3841 allowed us to identify a gene, named *hupE2*, encoding a product 50% identical to HupE. Subsequently, single and double mutants in *hupE* and *hupE2* were generated in UPM791 strain and also in SPF25 strain, a UPM791 derivative modified for over expression of hydrogenase in free-living microaerobic conditions [2]. The use of these mutants revealed that *hupE* and *hupE2* were essential for hydrogenase activity of *R. leguminosarum* in free-living microaerobic conditions, and that the absence of functional *hupE* and *hupE2* genes could be replaced by the addition of nickel. These results add evidence for a role of HupE in the transport of the nickel required for hydrogenase synthesis.

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T13. The hydrogenases of *Thiocapsa roseopersicina.*

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The purple sulfur phototrophic bacterium, *Thiocapsa roseopersicina* BBS contains several NiFe hydrogenases. One of these enzymes (HynSL) is remarkably stable and can be used for practical applications. The expression of *hynSL* is upregulated under anaerobic conditions with the participation of FNR. The *hupR* gene was identified in the gene cluster downstream from *hupSL*. Although a typical H_2 sensor (*hupUV*) and two-component regulator (*hupR, hupT*) are present in *T. roseopersicina*, the system is. Introduction of actively expressed *hupTUV* restored hydrogen dependent regulation of the *hupSL* gene expression. A third hydrogenase activity was located in the soluble fraction and was analogous to the NAD-reducing hydrogenases of cyanobacteria. The *hoxEFUYH* genes are transcribed together. *HoxE* is needed for the *in vivo* electron flow to and from the soluble hydrogenase. Some of the accessory genes were identified using random mutagenesis. The HupD, HynD and HoxD gene products correspond to the specific proteases of the three NiFe hydrogenases, respectively. The specificity of the *hupK*, *hypC1* and *hypC2* genes were investigated. The inactivation of the *hupK* gene yielded a nitrogenase independent phototrophic H_2 production. Both HypC proteins are needed for the biosynthesis of each NiFe hydrogenase.

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T14. Genetics and regulation of hydrogenases from green algae

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Recently, it was shown that [Fe]-hydrogenases are widely distributed among green algae and some anaerobic living protists. The simple structured enzymes (HydA) of algae catalyse H_2-evolution with a high specific activity that is comparable to the more complex [Fe]-hydrogenases of prokaryotes. The monomeric enzymes of about 50 kDa contain signal peptide sequences which route the nucleus encoded proteins to the chloroplast.
Two different variants of the *hydA* gene could be identified in several Chlorophycean species. The regulation of *hydA* expression takes place at the transcriptional level only in the absence of oxygen. We investigated the influence of O_2 on the transcriptional regulation of the *hydA* gene by reporter gene studies.
Besides hydrogen, the algae produce fermentative products like formate, ethanol, acetate and glycerol under anaerobic conditions. New results of the complex fermentative metabolism which is similar to mixed acid fermentation of *Escherichia coli* are presented.

T15. Formate and its Role in Hydrogen Production in *E. coli*

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In *E. coli* formate is the substrate for hydrogen evolution by the formate hydrogenlyase (FHL) complex. Formate is also essential for expression of the structural genes that form the FHL complex and it enhances expression of the genes encoding the ancillary proteins required for Ni-Fe cofactor biosynthesis and hydrogenase maturation. This enhancement in expression ensures that there is sufficient capacity to allow maximal FHL synthesis and consequently hydrogen production. Hydrogen evolution only occurs during fermentation. The principle source of formate in the anaerobic *E. coli* cell is from the pyruvate formate-lyase (PFL) reaction. During fermentation pyruvate is exclusively catabolised by PFL. Under these conditions every molecule of pyruvate catabolised generates one molecule of formate. Formate is also generated when *E. coli* cells respire anaerobically, for example with nitrate. Under these conditions expression of the FHL pathway is not induced. The formate generated is transported from the cytoplasm into the periplasm where formate dehydrogenases (FDH), particularly FDH-N, oxidise it to carbon dioxide and the reducing equivalents are linked to nitrate reduction and energy generation. When the nitrate is expended, any residual formate re-enters the cell where it can then induce FHL synthesis and resume hydrogen production. This compartmentalisation of a substrate helps govern which pathways the cell utilises to gain maximal energetic benefit from the substrate. The control of formate synthesis, transport and metabolism will be discussed in the light of its role as a key regulatory molecule.

T16. Novel Fe-hydrogenases from the rumen ciliate metagenome.

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The rumen of a cow hosts up to 10^{11} anaerobic ciliates representing more than 20 morphospecies and, potentially, several hundred ribotypes. All these ciliates are monophyletic and are believed to host hydrogenosomes. Using a metagenomic approach we were able to amplify the H-clusters of their Fe-hydrogenases. Preliminary phylogenetic analysis of some ten of them reveals that they cluster with all known eukaryotic Fe-hydrogenases including the NARF's. Only the complex [Fe]-hydrogenase of the anaerobic ciliate *N. ovalis* from the hindgut of cockroaches does not belong to this cluster suggesting an acquisition of its hydrogenase module from delta proteobacteria, and its covalently linked *nuoE,F* modules from beta proteobacteria, respectively. This interpretation is supported by the identification of the "mitochondrial" paralogues (24 kD and 51 kD) of the *nuo E,F* ORF's in the hydrogenosomes of *N. ovalis*.

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T17. Interacting Regulatory Networks in the Facultative Photosynthetic Bacterium, *Rhodobacter sphaeroides* 2.4.1

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Rhodobacter sphaeroides is capable of a wide diversity of growth modes depending upon environmental conditions. Adaptation to changes in the environment necessitate the rapid installation, but readily reversible, of regulatory schemes for the generation of energy and reducing equivalents. At a functional level, the cbb3 cytochrome c oxidase, is able to monitor the flow of reductant both aerobically and anaerobically, and is capable of integrating redox signals originating from diverse cellular pathways. These redox signals appear to be interpreted by different regulatory networks capable of controlling numerous metabolic functions, such as, photosynthesis, nitrogen and carbon dioxide fixation, as well as aerobic and anaerobic respiration [1]. Regulatory systems known to implement cbb3 generated signals involve the Prr two-component activation system [2], the PpsR/AppA repressor/anti-repressor system, FnrL and the RdxB redox cascade [3]. The Prr system alone controls, directly or indirectly, ~20% of the genome under anaerobic, dark conditions. Reductant flow through the cbb3 under anaerobic conditions appears to be monitored through the ratio of the carotenoids, spheroidene/spheroidenone. Overall, this regulatory network permeates nearly all aspects of cellular metabolism and when considered en toto, has the ability to monitor an infinitely variable input and to provide an output display for the maintenance of both cellular homeostasis and adaptation.

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T18. New Mechanistic Insight into the Oxygen Sensitivities of [NiFe]-Hydrogenases from Novel Electrochemical Experiments: Implications for Future Energy Technologies.

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The [NiFe] hydrogenases from *Allochromatium vinosum* and other bacteria can be adsorbed onto graphite electrodes at which they catalyze the electrochemical oxidation of H_2 at remarkable rates. Aside from exciting implications for future energy technologies, this makes possible novel kinetic experiments, featuring a rotating disc electrode, in which reactions of the active sites are initiated, controlled and monitored by stepping the electrode potential between different values. Over the course of a day, numerous experiments can be carried out on the same minuscule sample of enzyme, while the enclosed headspace is exchanged between O_2, N_2, CO and other gases. We have used this technique to determine the mechanism of formation of different inactive states of hydrogenases that are produced by exposure to O_2, as well as mechanisms of re-activation involving electrons and H_2. The studies reveal the role of H_2 and CO in activating the 'Unready' state (Ni-A) and provide us with a powerful way to apply and appraise hydrogenases for technologies such as fuel cells.

T19. Approaches to Developing Biological H_2 Photoproducing Organisms/Processes

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The availability of organisms capable of using sunlight energy to produce H_2 gas from water simultaneously with O_2 evolution provides a unique opportunity to develop renewable bioprocesses for energy production. However, the inhibitory effect of O_2 on H_2-producing hydrogenase enzymes represents a major technological challenge that needs to be addressed first. In collaboration with NREL's Computational Sciences Center and the Beckman Institute at the University of Illinois, we have carried out simulations of O_2-gas diffusion in an [Fe]-hydrogenase, which confirmed our hypothesis that O_2 gas accesses the enzyme's catalytic center through a finite number of paths, including the previously described hydrophobic gas channel. The computational model is being used to identify hydrogenase mutations that could hinder O_2 access and to further examine the relationship between structural properties of hydrogenases and their effect on O_2 inhibition. Selected mutations are being expressed in a heterologous *E. coli* system by co-transformation of the [Fe]-hydrogenase structural gene with the newly discovered hydrogenase assembly genes *hydEF* and *hydG*. A second approach involves the generation of a recombinant cyanobacterium that expresses an O_2-tolerant NiFe hydrogenase from the CO-oxidizing photosynthetic bacterium *Rubrivivax gelatinosus*. Preliminary work has been done on the construction of an expression plasmid containing all the genes essential for *Rubrivivax* hydrogenase function. Finally, our third approach is based on the selective effect of sulfate deprivation on photosynthetic water oxidation/O_2 evolution activity, which results in cellular anaerobiosis and induction of hydrogenase activity. Advances in all three approaches demonstrate that it is possible to creatively address the hydrogenase O_2-sensitivity problem and significantly impact the development of photosynthetic organisms as efficient sources of renewable H_2 gas.

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T20. Bioelectrocatalysis by hydrogenases. Towards hydrogen enzyme fuel electrodes.

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Molecular hydrogen is considered nowadays as the most promising chemical fuel, in particular for fuel cells. Fuel electrodes use platinum (or platinum metals) as electrocatalysts. There are, however, crucial problems, which make impossible wide applications of fuel cells in future: (i) cost and availability problem, (ii) poisoning by fuel impurities, (iii) lack of selectivity. Hydrogen fuel electrodes based on different hydrogenases immobilized directly on carbon filament material have been made. The enzyme electrodes are operated according to electron tunneling between the enzyme active site and the electrode support; this mechanism is called direct bioelectrocatalysis. After immersion of the enzyme electrode in neutral buffer solution saturated with molecular hydrogen, the equilibrium hydrogen potential is achieved. The enzyme electrode is characterized by high values of positive current in positive potential region. Hydrogen enzyme electrodes operated in neutral solutions are able to achieve electrocatalytic activities similar to that of platinum-based fuel electrodes in sulfuric acid. In neutral media electrocatalytic activity of platinum electrodes is decreased in average 100 times, which makes them incomparably less active. Hydrogenase enzyme electrode tested under different H_2 - CO mixtures showed no recognizable inhibition up to CO content of 0.1%. Only under 1% of CO the rate of hydrogen oxidation was decreased by approximately 10%. However, even after exposing to pure CO the hydrogenase electrode recovers 100% of its activity as soon as the atmosphere is changed back to hydrogen. It is possible to conclude, that biocatalysis is a valuable alternative to the catalysis by noble metals in respect to development of fuel cells.

T21. Biochemical and Process Optimisation Challenges for Fermentative Hydrogen Production.

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Hydrogen can be produced biologically by photosynthetic microrganisms or fermentative anaerobes. A number of fermentative anaerobes are known to produce hydrogen. These include mesophilic species such as *Enterobacter*, *Bacillus*, *Escherichia coli* and *Clostridium*, and extreme thermophiles such as *Calicellulosiruptor saccharolyticus* and *Thermoga elfii*. Although fermentative hydrogen production may offer a number of advantages over photosynthetic production, the biochemistry of fermentative hydrogen producing species and in particular the hydrogenase system has been relatively little studied when compared to photosynthetic hydrogen producing species. A theoretical maximum hydrogen yield of 4 mol H_2/mol glucose from fermentative anaerobes is predicted but in practice the hydrogen yield from fermentative anaerobes can vary from 1.61-2.36 mol H_2/mol glucose in *Clostridium* species to 3.3 mol H_2/mol glucose from the extreme thermophiles such as *C. saccharolyticus*. The hydrogen yield from fermentative anaerobes is influenced by a number of biochemical factors including pH, product inhibition, metabolic shift, intracellular and extracellular redox state. The influence that these biochemical parameters have on the hydrogen yield in particular on *Clostridium* based hydrogen production systems will be reviewed and the implications for process optimisation discussed.

T22. Applications of bacterial hydrogenases in waste decontamination, manufacture of novel bionanocatalysts and in sustainable energy

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The ability of *Escherichia coli* to reduce Tc(VII) to insoluble Tc(IV) was attributed to the activity of the hydrogenase-3 component of the formate hydrogenlyase complex. Decontamination of Tc(VII) was also achieved by *Desulfovibrio desulfuricans*, and this reductase activity also extended to the reduction and recovery of precious metals from wastes at the expense of H_2. Pd(II) was reduced to Pd(0) which was held as biotemplated and supported metallic nanocrystals on the cell surface. Bio-Pd(0) was ferromagnetic, attributable to a subset of 5 nm bionanoparticles which had a catalytic activity comparable to a commercial supported metal catalyst in a standard hydrogenation reaction, in addition to a novel ability to reductively dehalogenate polychlorinated biphenyls, which was not catalysed by chemically-reduced Pd(0). The catalytic activity of Bio-Pd(0) also extended to its use as an electrode material for a low temperature proton exchange membrane fuel cell, which makes electricity from hydrogen. The hydrogenase-3 activity of *E. coli* was also used synthetically in the generation of hydrogen by fermentation of industrial sugar wastes, using an upregulated mutant. Bio-H_2 from the overproducing strain was used as (a) the feed for biomanufacture of the Bio-Pd(0) bionanocatalyst, (b) for the bionanocatalyst-mediated decontamination of Cr(VI) and (c) as a H_2 supply for electricity generation using a PEM fuel cell. A test electrical load was supported by the direct use of off-gas from the industrial waste fermentation without need for gas filtration and without fuel cell catalyst poisoning.

T23. Roles of Hydrogen Oxidation in Pathogenesis by Infectious Bacteria

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Pathogenic *Helicobacter* species have the ability to use H_2 via a respiratory hydrogenase, and it was demonstrated that the gas is present in the stomach (the area colonized by the ulcer-causing bacterium Helicobacter pylori) of live animals [1,2]. Mutant strains of *H. pylori* are deficient in colonizing mice compared the parent strain [1]. Based on available annotated gene sequence information the enteric pathogen *Salmonella*, like other enteric bacteria, contains three putative membrane-associated H_2-using hydrogenase enzymes [2]. From analysis of gene-targeted mutants we conclude each of the 3 membrane bound hydrogenases of *Salmonella enterica* serovar Typhimurium are coupled to a respiratory pathway. Cells suspended in phosphate buffered saline consumed two moles of H_2 per mole of O_2 used in the H_2/O_2 respiratory pathway. From microelectrode probe measurements on live mice we detected H_2 within the tissues (liver and spleen) that are colonized by the enteric bacterium. The half-saturation affinity of *Salmonella typhimurium* for H_2 is only 2.2 μM, so it is expected that (H_2-utilizing) hydrogenase enzymes are saturated with the reducing substrate in vivo. All 3 hydrogenase enzymes contribute to virulence of the bacterium in a typhoid fever-mouse model. The introduced mutations were demonstrated to be non-polar. The combined removal of all 3 hydrogenases resulted in a strain that is avirulent and (in contrast to the parent strain) one that is unable to pass the intestinal tract to invade liver or spleen tissue. We conclude H_2 utilization in a respiratory fashion is required for energy production to permit *Salmonella* growth and subsequent virulence during infection.

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T24. The hydrogenase-like Nar1p is essential for maturation of cytosolic and nuclear iron-sulphur proteins.

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The genome of the yeast *Saccharomyces cerevisiae* encodes the essential protein Nar1p that is conserved in virtually all eukaryotes and exhibits striking sequence similarity to bacterial iron-only hydrogenases. A human homologue of Nar1p was shown previously to bind prenylated prelamin A in the nucleus. However, yeast neither exhibits hydrogenase activity nor contains nuclear lamins. Here, we demonstrate that Nar1p is predominantly located in the cytosol and contains two adjacent iron-sulphur (Fe/S) clusters. Assembly of its Fe/S clusters crucially depends on components of the mitochondrial Fe/S cluster biosynthesis apparatus such as the cysteine desulphurase Nfs1p, the ferredoxin Yah1p and the ABC transporter Atm1p. Using functional studies *in vivo*, we show that Nar1p is required for maturation of cytosolic and nuclear, but not of mitochondrial, Fe/S proteins. Nar1p-depleted cells do not accumulate iron in mitochondria, distinguishing these cells from mutants in components of the mitochondrial Fe/S cluster biosynthesis apparatus. In conclusion, Nar1p represents a crucial, novel component of the emerging cytosolic Fe/S protein assembly machinery that catalyses an essential and ancient process in eukaryotes.

T25. Biological Iron-Sulfur Cluster Formation

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Iron-sulfur clusters are one of the most ubiquitous prosthetic groups in nature and they play crucial roles in fundamental life processes such as respiration, photosynthesis and nitrogen fixation. Work performed in many laboratories over the past decade has resulted in the identification and characterization of a suite of proteins required for biological [Fe-S] cluster formation. Although there now appear to be several variations, all mechanisms appear to be unified by the activation of cysteine sulfur and the use of an [Fe-S] cluster-scaffolding protein. We have developed an in vitro system for the rapid, effective and efficient activation of the nitrogenase Fe protein. In more recent work we have developed a system for the controlled expression of [Fe-S] cluster biosynthetic components in *Azotobacter vinelandii* and used this system to evaluate the functions of individual players in the process of [Fe-S] cluster biogenesis.

T26.The Cyanide Ligands of [NiFe] Hydrogenases in *E. coli*: From HypE to the HypC x HypD complex

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The active site of [NiFe] hydrogenases consists of a bimetallic center to which three non-protein ligands are bound in the form NiFeCO(CN)2. At least in *E. coli* the cyanide is derived from carbamoylphosphate and is synthesised at the HypE protein to which the carbamoyl moiety has been transferred by the HypF protein [1]. As carbamoylphosphate deficient strains accumulate a protein complex between HypC and HypD [2], a transfer of the cyanide to this complex had been proposed. To follow this assumption experimentally the accessory proteins (HypE, HypF) or protein complexes (HypCxHypD) were purified. The biosynthesis and transfer of the cyanide ligands was assessed in vitro using ^{14}C-labeled carbamoylphosphate. It was found that at least three of the accessory proteins, namely HypC, HypD and HypE are able to form a ternary complex. The preparation of ^{14}C-labeled cyanide bound to HypE and subsequent addition of purified HypC x HypD complex led to the transfer of the cyanide to this complex but not to free HypC and HypD. The conclusion drawn is that the cyanide ligand, synthesised by HypE is not directly handed over to the hydrogenase large subunit. It rather indicates that it is the HypC x HypD complex to which the cyanides are attached first. As the stability properties of the cyanated HypC x HypD complex are not in favour of a protein-thiocyanate linkage (as on the HypE protein) it is speculated that the HypC x HypD complex is the scaffold at which the iron accepts the ligands before being transferred to the large subunit.

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T27. Maturation and biodiversity of hydrogenases from legume endosymbiotic bacteria.

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Several endosymbiotic bacteria from the *Rhizobium* group express hydrogenase activity in the legume nodule, thus allowing the recycling of hydrogen evolved by nitrogenase. Two such Hup systems, those from *Rhizobium leguminosarum* bv. viciae and *Bradyrhizobium japonicum*, have been studied in detail [1]. We have studied different factors affecting the maturation of *R. leguminosarum* hydrogenase. As in other bacteria, translocation of *R. leguminosarum* hydrogenase is dependent on the Tat (Twin-arginine translocation) system [2]. This system allows the export of the HupSL heterodimer according to a "hitchhiker" model [3]. In the case of *R. leguminosarum* a high number of proteins, apart from HupS, are predicted to depend on the Tat system for export. We have found evidence that not only the hydrogenase, but also components of the respiratory chain supporting hydrogen oxidation are dependent on the Tat system. The role of other proteins in the maturation of hydrogenase subunits will be also considered.

Another line of work in our laboratory deals with the analysis of biodiversity of hydrogenases from endosymbiotic bacteria. Cloning and sequencing of the Hup systems from *Bradyrhizobium* sp strains nodulating Vigna and Lupinus, and from *Azorhizobium caulinodans*, has revealed a significant degree of diversity of hup genes within this bacterial group. Analysis of the relationship with Hup systems from other bacteria suggests that phylogenetically distinct hydrogenase gene clusters have been incorporated into this bacterial group.

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T28. The multistep maturation pathway of membrane-bound [NiFe] hydrogenase

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Membrane-bound [NiFe] hydrogenases undergo a highly complex maturation process which involves metallocenter incorporation, specific proteolytic cleavage, subunit oligomerisation, Tat-mediated translocation and membrane attachment.
Analysis of specific requirements of the membrane-bound hydrogenase of *Ralstonia eutropha* is hampered by the fact that two additional [NiFe] hydrogenases, the NAD-reducing soluble hydrogenase (SH) and the H_2-sensing regulatory hydrogenase (RH), share at least the pleiotropic, catalytic site-forming Hyp proteins with the MBH. In order to study the MBH-specific maturation process, we subcloned a set of 21 genes comprising the MBH structural genes, *hyp* genes, specific auxiliary genes as well as regulatory genes on a broad host range plasmid. The resulting plasmid, designated pLO6, permits synthesis of catalytically active MBH in a *R. eutropha* derivative lacking the hydrogenase-encoding megaplasmid pHG1. Introduction of this genetically easily modifiable plasmid in well-defined mutant backgrounds allows the systematic analysis of MBH precursor proteins arrested in various maturation steps. We show that the MBH small subunit precursor, which carries the N-terminal Tat-leader peptide, is associated with specific proteins until a fully processed catalytic site-containing large subunit is available to form a transport-competent MBH dimer. The effect of site-directed *tat* gene mutations on MBH biosynthesis will also be discussed.

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T29.Identification of the [Fe]-hydrogenase assembly genes, *HydEF* and *HydG*, in *Chlamydomonas reinhardtii*

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The *hydEF* and *hydG* genes are required to assemble an active [Fe]-hydrogenase in *Chlamydomonas reinhardtii*. The *hydEF* gene is disrupted in the *C. reinhardtii* hydEF-1 mutant, which was identified by screening a library of random insertional mutants for clones that are unable to produce H_2. The hydEF-1 mutant transcribes both the *C. reinhardtii* hydrogenase structural genes, *hydA1* and *hydA2*, and accumulates full-length hydrogenase protein. However, the mutant is incapable of assembling an active [Fe]-hydrogenase and H_2-production activity is not observed. The HydEF protein contains two unique domains, which are homologous to two distinct prokaryotic proteins, HydE and HydF that are found exclusively in organisms containing [Fe]-hydrogenase. In the *C. reinhardtii* genome, the *hydEF* gene is adjacent to another hydrogenase-related gene, *hydG*. All organisms with [Fe]-hydrogenase and sequenced genomes contain homologues of *hydE*, *hydF* and *hydG*, which were previously of unknown function. Within several prokaryotic genomes, *HydE*, *hydF* and *hydG* are found in putative operons with [Fe]-hydrogenase structural genes. Both *hydE* and *hydG* belong to the emerging Radical SAM superfamily of proteins. We demonstrate here that HydEF and HydG function in the assembly of [Fe]-hydrogenase. Northern blot analysis indicates that mRNA transcripts for both the *hydEF* and *hydG* genes are anaerobically induced concomitantly with the two *C. reinhardtii* [Fe]-hydrogenase genes. Complementation of the hydEF-1 mutant with the wild-type *hydEF* gene restores hydrogenase activity. Moreover, the co-expression of the *C. reinhardtii hydEF*, hydG and *hydA1* genes in *Escherichia coli* results in the formation of an active HydA1 enzyme in this bacterium. This represents the first information on the nature of the accessory genes required for the maturation of an active [Fe]-hydrogenase.

This work was supported by the Division of Energy Biosciences, Office of Science, U.S. Department of Energy, and by the Hydrogen, Fuel Cells, and Infrastructure Technologies Program, U.S. Department of Energy

T30. The Tat protein transport system required for the biogenesis of extracytoplasmic hydrogenases

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Most uptake hydrogenases are localized to the extracytoplasmic compartment of the bacterial cell. Their biosynthesis therefore involves a protein export step. It is now clear that extracytoplasmic hydrogenases of both the [Ni] and [Fe] classes are translocated by the recently-discovered Tat protein transport system rather than the better-known Sec pathway. The Tat apparatus, in contrast to the Sec complex, transports folded substrates. In the case of the hydrogenases this allows both cofactor insertion and subunit association in the cytoplasm prior to transport. Proteins are targeted to the Tat pathway by amino-terminal signal peptides harbouring consecutive, invariant, arginine residues within an S-R-R-x-F-L-K consensus motif. The Tat system faces a particularly challenging mechanistic task. It must provide a transmembrane channel that is large enough to allow the passage of structured macromolecular substrates of up to 60Å in diameter while maintaining the impermeability of the membrane to ions, including protons. In addition the Tat transporter transduces the energy of the transmembrane proton electrochemical gradient to effect unidirectional movement of the substrate through the channel. I will review current knowledge of the Tat pathway and present our recent data on structure-function analysis of the Tat transporter of *Escherichia coli*.

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P1-1. Structural Models for the Hoxair State of Fe-only Hydrogenases

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Previous structural models for the Fe-only hydrogenases focused on simple substituted derivatives of $Fe_2(SR)2(CO)_6$, e.g. $Fe_2(SR)_2(CO)_4L_2$ (L = CN-, PMe_3) and their protonated derivatives. Species such as $[Fe_2(S_2C_3H_6)(CN)(PMe_3)(CO)_4]$- catalyze the reduction of protons to dihydrogen.[1] In this project we set out to synthesize diferrous dithiolate models of the more oxidized forms of the Fe-only active site, specifically Hoxair. The poster will survey our work on the $Fe2II(SR)_2(CO)x(L)_{7-x}$ species, which contain the previously elusive bridging carbonyl moiety. Such species arise from the "oxidative decarbonylation" of $[Fe(I)]_2$ species. Initial experiments of this reaction gave the $[Fe_2(SR)_2(CNMe)_7]_+$ derivatives.[2] Further refinement of experimental variables has allowed the synthesis of models with more realistic ligand sets, e.g. $Fe_2(SR)_2(m\text{-}CO)(CN)_2(L)_2(CO)_2$.

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P1-2. Bioinspired Ligands and Catalysts: NiN_2S_2 Moieties as Metallothiolate Ligands

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The coordination chemistry of mercapto groups, derived from cysteine residues on proteins (as in [NiFe]H_2ase), or from the bidentate ligand, -$SCH_2CH_2CH_2S$- or -SCH_2NHCH_2S- cofactor (as in [FeFe]H_2ase), is of major importance in the construction of hydrogenase enzyme active sites. Typical of small molecule precedents, a dominant theme in the structures of the active sites is the formation of bridges between metal ions by mercapto sulfurs. We have employed the N_2S_2 ligand, bis-mercaptoethanediaza-cyclooctane, as a convenient agent for the formation of monomeric nickel(II) square planar complexes, serving as a precursor for heterometallic complexes of some relation to the [NiFe] H_2ase active site. Even more appropriate is the connection between the distal nickel of the acetyl coA synthase and square planar NiN_2S_2 complexes. An open question, particularly in the latter case, is the extent to which the NiN_2S_2 moiety might serve as a metallodithiolate ligand, an innocent ligand, with distinguishing steric and electronic donor properties which tailor the reactivity of the sulfur-bound exogenous metal as would classical ligands of the coordination chemist. In addition to providing understanding for mechanistic features of the enzyme active sites, the biocatalysts serve as inspiration to the synthesis and development of artificial catalysts. The goal of this research has been to establish parameters that characterize the donor ability of NiN_2S_2 complexes for comparison to classical diimine and diphosphine ligands, and for their development into a new class of chelating ligands for use in organometallic chemistry. Thus we have prepared a series of $(NiN_2S_2)W(CO)_4$ complexes and through the reporter capability of the CO ligand ν (CO) vibrational spectroscopy) determined that the donor ability of the nickel dithiolates is similar to that of the bipyridyl ligand, and greater than diphosphine ligands. The structural scope of this series of ligands is extensive; polynuclear paddlewheel-type complexes and new heterobimetallic complexes will be presented.

P1-3. Diruthenium Models of Fe-only Hydrogenase Enzyme Active Site

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Although the diiron active site of the Fe-only hydrogenase has been studied extensively by synthetic modeling and theoretical experiments, no complexes with terminal hydride or dihydrogen ligands have been produced.[1,2] Subferrous species of the type $[Fe_2(SR)_2(CO)_4LL']Z$ ((i) L = L' = CN-, z = -2; (ii) L = PMe_3, L' = CN-, z = -1, (iii) L = L= PR_3, z = 0) protonate readily, but the site of attack is the Fe-Fe bond.[3-5] Interactions between diiron thiolates and dihydrogen is implicated in the photocatalyzed exchange between H_2 and D_2O involving $[Fe_2(S_2C_3H_6)(\mu\text{-}X)(CO)_4(PMe_3)_2]+$[6]. We have addressed this and related questions through studies on diruthenium system of $Ru_2(S_2C_3H_6)(CO)_4(PCy_3)_2$ (Cy = C_6H_{11}). This species reacts with H_2 under photolytic conditions to produce $Ru_2(S_2C_3H_6)(\mu\text{-}H)(H)(CO)_3(PCy_3)_2$. X-ray crystallography reveals the presence of both bridging and terminal hydrides. The dihydride complex, $Ru_2(S2C_3H_6)(\mu\text{-}H)(H)(CO)_3(PCy_3)_2$, reacts with a proton source to yield $[Ru_2(S2C_3H_6)(\mu\text{-}H)(H_2)(CO)_3(PCy_3)2]+$, the first example of a dihydrogen ligand in a synthetic model of the Fe-only hydrogenase. The corresponding $[Ru_2(S_2C_3H_6)(\mu\text{-}H)(HD)(CO)_3(PCy_3)_2]+$ was also characterized (JHD = 31 Hz).

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P1-4. Functionalized Structural Models of Fe-Only Hydrogenase Active Sites

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Subferrous models of the hydrogenase active site bearing functionalized dithiolate straps will be presented [1, 2, 3]. Previously we have synthesized models containing azadithiolate straps, with the formula $Fe_2[(SCH_2)_2NR](CO)_4(L)_2$, where L = CO, CN-, via Mannich-like condensations [4, 5, 6]. We have taken a closer look at the condensation reaction and its limitations. The repertoire of azadithiolates has been extended to include functionalities such as alcohols, amides, and esters. A second class of functionalized models features a dihydroasparagusic acid moiety as the dithiolate source. This coligand installs a carboxylic acid within bonding distance of the metal center. Examination of the pendant carboxylic acid's chemistry concerning metal coordination, amino acid derivatization, and electrocatalysis will be described.

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P1-5. Dithiolate-Bridged Di-Iron Carbonyls: Good Models of the H-Cluster ?

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In addition to the obvious structural relationship between the {2Fe} subsite of the [Fe]-hydrogenase H-cluster, dithiolate-bridged di-iron carbonyl compounds are electrocatalytic in terms of proton reduction. The reduction chemistry of these compounds is rich and is highly sensitive to the identity of the bridging ligand. The details of this chemistry have been examined y a range of spectroscopic and spectroelectrochemical techniques. Details of the structures (such as the Fe-Fe bond length) of several of the reactive species have been obtained from EXAFS analysis, where the presence of a bonding Fe-Fe interaction is important in terms of structure deduction.
In the presence of moderately strong acids protonation of the propanddithiolate-bridged compound occurs at the one electron reduced level and hydrogen elimination occurs from the two-electron reduced doubly protonated form. A second path leading to hydrogen evolution involves further reduction and this is evident in reactions carried out at higher acid concentrations. As for the H-cluster, the activity of the catalyst is reduced by elevated concentration of CO. This is explained in terms of a side reaction that leads, ultimately to a CO-bridged di-iron product. The ethanedithiolate-bridged analogue does not suffer CO inhibition. The behaviour of the thiolate-bridged compounds contrasts with that of the phosphido-bridged analogues, where protonation occurs following two-electron reduction. In this case terminally-bound hydrides are formed, however hydrogen evolution does not occur until the two-electron reduced, doubly protonated compound is further reduced. Structures of the products formed following electron or electron/proton reaction are proposed in the light of spectroscopic and EXAFS results and these form the basis of mechanisms proposed for the electrocatalytic proton reduction reactions.

P1-6. Synergy Between Theory and Experiment as Applied to [Fe]H_2ase Active Site Models

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Of importance to applications of the lessons learned from hydrogenases is the development of functional models into working catalysts for hydrogen uptake and hydrogen production. Computational chemistry provides a link between the enzyme active site and inorganic/organometallic synthetic analogues. Our projects have focussed on 1) the computation of viable reaction pathways for isotopic scrambling of H_2/D_2O, D_2/H_2O, and D_2/H_2 mixtures using dinuclear FeIIFeII complexes as catalysts and 2) the role of asymmetry in creating a flexible coordination sphere about Fe in FeIFeI complexes which serve as solution electrocatalysts for H_2 production. For the former, density functional theory (DFT) calculations were used to examine dihydrogen cleavage by a photochemically generated h_2-D_2 complex of the form {(m-H)(m-$S(CH_2)2S$)[$Fe(CO)_2PH_3$][$Fe(CO)(PH_3)(h_2\text{-}D_2)$]}1+. The calculations suggest that a reasonable path for D_2/H_2O scrambling involves deprotonation of bound D_2 by a water cluster to generate a dideuteride species. This complex may then reductively eliminate HD to generate a h_2-HD bound FeIFeI complex. Protonation of the Fe-Fe bond by $\{D(H_2O)_n\}$+ affords D_2/m-H exchange. For the latter, the assumption is that an electron-rich "open site" on iron is required prior to H^+ uptake and reduction. DFT computations were used to examine how the nature of L in a series of mono-substituted complexes of the form {(m-SRS)[$Fe(CO)_3$][$Fe(CO)_2L$]$_2$}n- effects intramolecular site exchange at the individual iron centers. The computed barriers to rotation of the $Fe(CO)_3$ and $Fe(CO)_2L$ units are shown to be directly related to the donor strength of L. As the donor strength of L is increased, the barrier to $Fe(CO)_3$ rotation decreases and the barrier to $Fe(CO)_2L$ rotation increases. Analysis of the calculated atomic charges shows that rotation of the the $Fe(CO)_3$ unit leads to transfer of electron density from the unrotated $Fe(CO)_2L$ unit to the rotated $Fe(CO)_3$ unit.

P1-7. Synthesis of {2Fe3S} cores related to the sub-site of [Fe]-Hydrogenase: towards total synthesis of the H-Cluster.

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Within all-iron hydrogenase, the reduction of protons takes place at the so-called "H-cluster", a six-iron assembly consisting of a [Fe4S4]-cluster linked by a thiolate cysteine bridge to a di-iron unit. The synthesis of {2Fe3S} compounds related to the sub-site will be described, including an activated thioacetyl di-iron unit. This latter compound provides a route for the construction of an assembly with structural features of the enzymic H-cluster.

P1-8. Pulsed Electron-Electron Double Resonance on Multi-Nuclear Metal Clusters: Assignment of Spin Projection Factors Based on the Dipolar Interaction

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The interaction between two paramagnetic metal centers, a [3Fe-4S]^+ cluster and a [NiFe] center, is investigated in the hydrogenase from *Desulfovibrio vulgaris* Miyazaki F by pulsed ELDOR (electron-electron double resonance). The distance between the metal centers is known from X-ray crystallography. The experimental dipolar spin-spin coupling deviates from the value expected for two point-dipoles located at the centers of the metal clusters. An extended spin-coupling model accounting for the spin distribution in the [3Fe-4S]^+ cluster yields the observed coupling under the assumption of a particular magnetic coupling scheme for the three Fe ions. These results demonstrate that pulsed ELDOR can be used to gain insight into the inner structure of a multi-nuclear metal cluster.

P2-1. Gas diffusion studies inside the *Cp*I [Fe]-hydrogenase

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The photobiological conversion of H_2O into H_2 by the green algae *Chlamydomonas* reinhardtii offers a promising system for the production of clean, renewable H_2. However, the quick deactivation of the catalytic site buried inside the algal [Fe]-hydrogenase by atmospheric O_2 poses a serious obstacle to an economical, large scale H_2 production system. All-atom computer simulations of the diffusion process of small gaseous inhibitors inside a hydrogenase protein can provide answers as to how to engineer the hydrogenase to be more robust against O_2 deactivation. We have carried out simulations of the diffusion of the O_2 inhibitor and the H_2 product inside a model of the homologous *Cp*I [Fe]-hydrogenase from *Clostridium pasteurianum* obtained from X-ray crystallography. Preliminary results suggest that O_2 can enter and exit the enzyme through a finite number of paths consisting of connected cavities. These areas of high O_2 occupancy during our simulations correlate well with pre-existing
"packing defects" present in the crystal structure. The much smaller H_2, on the other hand, can more freely diffuse throughout the protein. These results suggest that, in theory, it is possible to increase the O_2 tolerance of [Fe]-hydrogenases by limiting O_2 diffusion without preventing the H_2 product from being released by the protein. By targeting mutations to the O_2 pathway-lining residues, O_2 diffusion within the protein could be reduced while H_2 outward diffusion would be unaffected. Eventually, we wish to extend the knowledge gained from modeling and simulating the bacterial *Cp*I [Fe]-hydrogenase towards engineering of the highly homologous [Fe]-hydrogenase from *Chlamydomonas reinhardtii*.

P2-2. Carbon monoxide as an intrinsic ligand to iron in the active site of the iron-sulfur cluster free hydrogenase (Hmd) as revealed by infrared spectroscopy

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The iron-sulfur cluster free hydrogenase, Hmd, from methanogenic archaea has recently been found to contain one iron associated tightly with an extractable cofactor of yet unknown structure.[1] Hmd was shown to be inhibited by carbon monoxide and cyanide and to be light sensitive, inactivated upon exposure to UV-A (320-400 nm) or blue light (400 -500 nm).[2] Enzyme purified in the dark exhibited an absorption spectrum with a maximum at approximately 360 nm. Fourier transform infrared spectra of the native enzyme showed two bands of almost equal intensity at 2011 and 1944 cm-1, interpreted as the stretching frequencies of 2 CO molecules bound to the same iron.[3] In white light, the IR absorption bands disappeared and the enzyme was bleached, loosing its absorption maximum at 360 nm. Infrared of Hmd inhibited with ^{12}CO, ^{13}CO, ^{12}CN-, and ^{13}CN- revealed an interaction of these diatomic molecules with the Fe center of the enzyme. Binding of exogenous CO and CN- was found to be mutually exclusive and reversible. The cofactor extracted from active Hmd was also light sensitive, showed a UV/visible spectrum similar to that of the active enzyme, and had two n(CO) bands at 2031 and 1972 cm-1 similar to those of active Hmd. The free cofactor showed no interaction or exchange with exogenous ^{12}CO or ^{13}CO.

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P2-3. EPR of ^{61}Ni-enriched [NiFe] hydrogenase from *Desulfovibrio vulgaris* Miyazaki F.

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Abstract: In this contribution, we examine the ^{61}Ni hyperfine interaction in the standard [NiFe] hydrogenase of *D. vulgaris* Miyazaki F by multifrequency Electron Paramagnetic Resonance (EPR) and Electron Electron Double Resonance (ELDOR) detected NMR at 34 GHz. The labelling was done by growing the culture in a 10L glass bottle fermenter in the minimal medium, with metals selectively removed, and enriched with an optimised concentration of ^{61}Ni as ^{61}NiCl2 (92% enriched). The EPR spectra of the protein thus obtained showed a ^{61}Ni incorporation of >90% and indicate that the built-in of the ^{61}Ni isotope is comparable in magnitude to the purity of the isotopically labelled starting material. Hyperfine coupling constants have been elucidated for all EPR active redox states (A, B, C and L). Besides EPR experiments, ENDOR measurements were attempted, but no ^{61}Ni signals could be detected. This is probably related to the very fast spin relaxation, which makes it very difficult to measure metal hyperfine interactions in general. With orientation-selected ELDOR detected NMR, another hyperfine resolving technique, signals were observed. The hyperfine interaction could thus be recorded and analysed with superiour resolution than in EPR. In parallel, density functional theory (DFT) calculations, using the B3LYP functional, a spin-unrestricted formalism and the triple zeta basis set from Ahlrichs *et al.* [1], have been performed on model systems and ^{61}Ni hyperfine parameters have been calculated. Experiment and theory are compared and good agreement is found. This indicates that the chosen models, already successful to describe ligand hyperfine interactions [2], correctly describe the wavefunction near nickel for the redox states as well.

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P2-4. IR spectroelectrochemical characterization of different redox states of [NiFe] hydrogenase from *Desulfovibrio vulgaris* Miyazaki F

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The different redox states Ni-B, Ni-SI, Ni-C and Ni-R of [NiFe] hydrogenase were investigated by IR spectroelectrochemistry. For all experiments we used a modified OTTLE cell which enables the application of a distinct redox potential to the enzyme solution containing a set of redox mediators. We were able to assign the characteristic CO and CN bands of the redox states for the first time for *D. vulgaris* hydrogenase showing the existence of one CO and two CN molecules as Fe ligands. Electrochemically controlled redox titrations allowed the determination of the midpoint potentials of the different redox equilibria at different pH values. With increasing pH the midpoint potential shifted to lower values. This variation was smaller than the theoretically expected value of (RT/nF)ln10 = 60 mV/pH unit with T=303 K and n=1 according to the Nernst equation. Two additional Ni-R states were detected representing subforms of the main Ni-R state. The difference between Ni-R and Ni-R´ is not yet clear. Additional Ni-R states are also detected in other hydrogenases. Here it is assumed that these subforms may differ in their protonation state from the main Ni-R form [1,2]. The two Ni-SI forms, Ni-SIr and Ni-SIa, were found to be in an acid-base equilibrium in which Ni-SIa is the protonated form. It is predominantly present in spectra at low pH. Two catalytically important processes, the inactivation process (oxidation of Ni-SI to Ni-B) and the activation process (reduction of Ni-A, Ni-B and Ni-SI), are investigated at different temperatures and pH values.

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P2-5. FTIR-spectroelectrochemical study of the activation and inactivation processes of [NiFe] hydrogenases: effects of solvent isotope replacement and site-directed mutagenesis

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The kinetics of the activation and anaerobic inactivation processes of *Desulfovibrio gigas* hydrogenase have been measured in D_2O by FTIR-spectroelectrochemistry. A primary kinetic solvent isotope effect was observed for the inactivation process but not for the activation step. The kinetics of these processes have been also measured after replacement of a glutamic residue placed near the active site of an analogous [NiFe] . hydrogenase from *Desulfovibrio fructosovorans*. Its replacement by a glutamine affected greatly the kinetics of the inactivation process but only slightly the activation process. The interpretation of the experimental results is that the rate-limiting step of anaerobic inactivation is the formation from water of a m-OH- bridge at the hydrogenase active site, and that glutamate 25 has a role in this step [1].

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P2-6. Biochemical and spectroscopic characterization of an isolated regulatory hydrogenase large subunit.

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[NiFe] hydrogenases are minimally heterodimers consisting of a large, active-site-harboring subunit and a small subunit containing at least one FeS cluster. Spectroscopic investigation of the iron in the [NiFe] active site is complicated, if not completely prevented, by interference from iron in the FeS clusters of the small subunit. Therefore it would be useful to examine the large subunit in the absence of the small subunit. Furthermore, such an isolated large subunit could serve as an irreplaceable tool for investigating the biosynthesis of the active site. Although it is known that six accessory proteins are necessary for assembly of the hydrogen activating site, the details of this biosynthetic pathway are yet to be elucidated. We have isolated and characterized the large subunit, HoxC, of the regulatory hydrogenase from *Ralstonia eutropha* without the corresponding small subunit HoxB. A deletion of nearly 50% of the hoxB gene ensured that only hoxC was expressed. Moreover, the fusion of a Strep-tag II to the N-terminus of HoxC allowed purification of HoxC-Strep in one step via affinity chromatography. The isolated HoxC-Strep subunit has been characterized spectroscopically and biochemically. Mass determination by size exclusion chromatography showed that HoxC-Strep was present in solution primarily as a dimer. Nickel autoradiography and XAS analysis determined that both the Ni and the Fe were incorporated into HoxC-Strep. Furthermore, the three diatomic ligands were detected by FTIR spectroscopy suggesting that, despite the absence of the small subunit, the active site was correctly incorporated. Although the [NiFe] center was apparently correctly assembled, no hydrogen oxidation activity could be detected. This suggests that although the hydrogen activating [NiFe] site is present in the large subunit the small subunit is nonetheless essential for hydrogen oxidation activity.

P2-7. Spectroscopic Characterization of Oxygen-Insensitive [NiFe] Hydrogenases.

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[NiFe] hydrogenases, which are active in the presence of dioxygen, are particularly interesting with respect to biomimetic and biotechnological applications. In the absence of crystallographic information, the molecular basis of O_2-insensitive hydrogen catalysis is barely understood. We have studied two O_2-insensitive [NiFe] hydrogenases from the bacterium *Ralstonia eutropha*. (1) The soluble, NAD-reducing hydrogenase (SH) provides reducing equivalents for CO_2 fixation; (2) the regulatory hydrogenase (RH) is a hydrogen sensor. Information on the atomic structure of the NiFe-cofactor and on structural and oxidation state changes of the redox cofactors (active-site [NiFe], [FeS] clusters) in the course of the catalytic cycle of mechanistic relevance was obtained from X-ray absorption spectroscopy (XAS), EPR and FTIR. The structural features of the Ni-site of the SH and of the RH [1-6] were studied during activation and catalysis. Both Hydrogenases revealed an unusual coordination of the Ni atom of the [NiFe] site; namely less than four thiol ligands [7, 8], at variance with standard hydrogenases. Pronounced structural changes at the [NiFe] cofactors during catalysis were detected and tentatively related to the mechanism of hydrogen sensing in the RH [7] and to the unusual catalytic cycle of hydrogen cleavage in the SH. First insights into the reasons for O_2-insensitive catalysis were obtained.

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P2-8. The Soluble Hydrogenase of *Ralstonia eutropha*. Adaptation of a Hydrogen-oxidizing Enzyme to Aerobic Operating Conditions.

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The soluble hydrogenase (SH) of *Ralstonia eutropha* is a [NiFe]-hydrogenase that can operate under aerobic conditions. It is a heterotetrameric enzyme with subunits HoxF (67 kDa), HoxH (55 kDa), HoxU (26 kDa) and HoxY (23 kDa). It comprises two functionally different, heterodimeric complexes. The HoxFU dimer is a NADH-dehydrogenase involved in the reduction of NAD^+, and the HoxHY dimer forms the hydrogenase module within the SH. Compared to standard [NiFe]-hydrogenases, the SH shows some clear differences. Firstly, the Ni-Fe active site consists of a $(CN)NiFe(CN)_3CO$ group instead of a $NiFe(CN)_2(CO)$ group. Secondly, the activity of the enzyme is not inhibited by O_2 or CO. In addition, the Ni in the SH is redox inactive in contrast to Ni in standard [NiFe]-hydrogenases. Further, the hydrogenase module is dependent for its function on an FMN group [2]. A possible mechanism of action of the SH will be presented [1-3]

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P2-9. A multisubunit membrane-bound [NiFe] hydrogenase and a NADH-dependent Fe-only hydrogenase in the fermenting bacterium *Thermoanaerobacter tengcongensis*

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Thermoanaerobacter tengcongensis is a thermophilic gram-positive bacterium able to dispose the reducing equivalents generated during the fermentation of glucose to acetate and CO_2 by reducing H^+ to H_2. H_2 formation in this organism was found to be catalyzed by a unique combination of hydrogenases: a ferredoxin-dependent [NiFe] hydrogenase and an NADH-dependent Fe-only hydrogenase. Both enzymes were purified and characterized. The tightly membrane-bound [NiFe] hydrogenase belongs to a small group of complex-I-related [NiFe] hydrogenases and has highest sequence similarity to Ech hydrogenase from *Methanosarcina barkeri*. A ferredoxin isolated from *T. tengcongensis* was identified as physiological substrate of this enzyme. The heterotetrameric Fe-only hydrogenase was isolated from the soluble fraction. It contained FMN and multiple iron-sulfur clusters, and exhibited a typical H-cluster EPR signal after autooxidation. The encoding genes are clustered in the *T. tengcongensis* genome. Sequence analysis predicted and kinetic studies confirmed that the enzyme is an NAD(H)-dependent Fe-only hydrogenase. When H_2 was allowed to accumulate in the culture, the fermentation was partially shifted to ethanol production. In cells grown at high hydrogen partial pressure ($p(H_2)$) the NADH-dependent hydrogenase activity was four-fold lower than in cells grown at low $p(H_2)$, whereas aldehyde dehydrogenase and alcohol dehydrogenase activities were higher in cells grown at elevated $p(H_2)$, which indicated a regulation in response to the $p(H_2)$.

Soboh, B., Linder, D. & Hedderich, R. (2004) Microbiology, in press

P2-10. Homologous/heterologous over-expression in *Clostridium acetobutylicum* and characterization of purified clostridial and algal Fe-only hydrogenases with high specific activity

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To study the structure-function relationships of Fe-hydrogenases, characterization of over-expressed purified native and modified Fe-hydrogenases is needed. Since the heterologous expression of Fe-only hydrogenases in *Escherichia coli* or in the cyanobacterium *Synechococcus* led to enzymes in an inactive form [1, 2, 3] or with low hydrogen evolution activity [4, 5], we developed a homologous/ heterologous over-expression and purification system functional in *Clostridium acetobutylicum*. C. acetobutylicum ATCC 824 was selected for the homologous over-expression of its Fe-only hydrogenase and for the heterologous over-expressions of the *Chlamydomonas reinhardtii* and *Scenedesmus obliquus* HydA1 Fe-only hydrogenases. The Fe-hydrogenases were over-expressed as Strep-tag II tagged proteins, purified by affinity chromatography and were fully active with high specific activities. Addition of the tag did not affect the electronic properties of the paramagnetic species within the protein. kinetic parameters showed that The natural substrate ferredoxin was a more suitable substrate for the *C. acetobutylicum* Fe-only hydrogenase compared to methyl viologen. Both algal hydrogenases accept not only plant type ferredoxin with a [2Fe-2S] cluster, but also [4Fe-4S] bacterial type ferredoxin as electron donors. This represents a breakthrough for the study of the structure-function relationships of Fe-only hydrogenases.

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P2-11. Structural analysis of the iron-sulfur cluster free hydrogenase Hmd from methanogenic archaea.

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Three types of hydrogenases are known to date. Two of these, the [NiFe]-hydrogenases and the [FeFe]- hydrogenases are iron-sulfur proteins. The third one, Hmd, does not contain iron-sulfur clusters and harbours a tightly bound cofactor that contains iron and that is required for Hmd activity [1]. The apoprotein of the enzyme from *Methanopyrus kandleri* was heterologously produced in *Escherichia coli*, purified and crystallized. The structure of the selenomethionine labelled apoprotein could be solved up to a resolution of 2.8 Å [2]. The preliminary model revealed that the enzyme is a homotetramer composed of two functional homodimers. Each monomer has two domains, an N-terminal domain (255 residues) with a fold typical for proteins with a dinucleotide binding site, and a C-terminal domain (94 residues) which in the homodimer is intertwined with the C-terminal domain of the second monomer.

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P2-12. The cofactor of the iron-sulfur cluster free hydrogenase Hmd: structure of the light-inactivation product.

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Hmd is a hydrogenase involved in methane formation from H_2 and CO_2 in many methanogenic archaea. This enzyme is overproduced in *Methanothermobacter marburgensis* when the cells are grown under nickel limiting conditions. The enzyme differs from all other hydrogenases in that it does not contain iron sulfur clusters. For activity, Hmd requires a tightly bound cofactor which can be released by unfolding the enzyme with urea in the presence mercaptoethanol [1]. The purified cofactor contained iron which remained bound even in the presence of 50 mM EDTA. Mössbauer spectroscopy indicated that the cofactor iron atom is most probably in a Fe2+ low spin state in the holoenzyme. Upon irradiation with UV-A/blue light, the cofactor was inactivated and the iron was released [2]. The cofactor was also temperature sensitive, the sensitivity being reduced in the presence of 2-mercaptoethanol. But even in the presence of 10 mM 2-mercaptoethanol, approximately 50% of its activity was lost in 1 hour at 50°C. The inactivated cofactor, with a molecular mass of 542 Da, was found to be a (6-carboxymethyl-3,5-dimethyl-2-pyridone-4-yl)-(5´-guanosyl) phosphate [3].

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P3-1. Characterization of [Fe]-hydrogenase structural elements affecting inhibitor sensitivity and catalysis

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Hydrogen production in the green alga *Chlamydomonas reinhardtii* is a strictly anaerobic process that can be coupled to photosynthesis under conditions of sulfur deprivation. Cells deprived of sulfur undergo a series of physiological events that cause reduced PSII activity, respiratory consumption of residual O_2, and establishment of an anaerobic state. Induction of hydrogenase gene expression and enzyme synthesis ultimately results in H_2-production. Although metabolic sequestering of O_2 overcomes the severe sensitivity of algal [Fe]-hydrogenase to O_2 inhibition, the overall H_2-production efficiency is reduced. To develop an efficient, large-scale, photobiological H_2-production system requires addressing the high O_2 sensitivity of algal [Fe]-hydrogenases. Engineering of algal [Fe]-hydrogenases designed with improved catalytic efficiency and reduced inhibitor sensitivity is one approach towards developing a more robust H_2-production system. We have investigated the structural and functional properties of algal [Fe]-hydrogenases and homologous bacterial [Fe]-hydrogenases in an effort to identify elements critical to O_2 sensitivity and proton transfer. The O_2-sensitivity studies are being complemented by a systematic computational approach aimed at identifying O_2-diffusion pathways in [Fe]-hydrogenase. The outcomes of these investigations are being used to guide an iterative design of modified [Fe]-hydrogenases with improved O_2 tolerance. Functional characterization of modified enzymes is being carried out in a recombinant [Fe]-hydrogenase expression system that co-expresses the algal [Fe]-hydrogenase assembly proteins. Initial experimental results on [Fe]-hydrogenase mutants with altered O_2 sensitivity will be reported. Previous structural studies of [Fe]-hydrogenase from *Clostridium pasteurianum* and *Desulfovibrio desulfuricans* identified several residues proposed to function in proton transfer to the catalytic site. One of these, a conserved cysteine located adjacent to the H-cluster, was mutated to a serine resulting in the loss of catalytic activity.

P3-2. NiFe hydrogenase active site biosynthesis: Hyp protein complexes in *Ralstonia eutropha*

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The mechanism of the biosynthesis of the NiFe hydrogenase active site, especially that of the biologically unprecedented, intrinsic CO and CN- ligands, is one of the most fascinating mysteries facing bioinorganic chemists. NiFe active site maturation requires six Hyp proteins: HypA-HypF. Data from *Escherichia coli* have demonstrated that HypE and HypF are able to synthesize a thiocarbamate residue at the C-terminus of HypE. It is proposed that this modification is transferred as cyanide from HypE to an Fe harbored in a HypC/HypD complex; however, the interaction of either HypC or HypD with HypE or HypF has yet to be experimentally demonstrated. Thus further experiments are necessary to elucidate the details of the transfer of diatomic ligands to Fe and the apo-large subunit. In this study, we investigate maturation of NiFe hydrogenases in *Ralstonia eutropha*, an organism that, unlike *E. coli*, produces hydrogenases under aerobic conditions. The synthesis of the diatomic ligands in this organism is of particular interest since the soluble hydrogenase from *R. eutropha* has been demonstrated to contain more CN- ligands than the standard enzymes. Using an affinity purification procedure, *R. eutropha* HypC and HypE were homologously expressed and purified from different genetic backgrounds as complexes with other hydrogenase-related proteins. HypE formed a stable complex with HypF. Additionally, HypE was observed in complexes with HypC and HypD but only if all three proteins were present. This suggests HypC and HypD function as a single entity with respect to HypE. The HypC/HypD/HypE complexes did not involve HypF but were stabilized by its presence in the cells. These results provide the first evidence linking the actions of HypC/HypD to HypE/HypF. A model for hydrogenase maturation in *R. eutropha* will be proposed in this light.

P3-3. Specific and pleiotropic hydrogenase accessory proteins in *Thiocapsa roseopersicina*

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There are, at least, two membrane-bound (HynSL and HupSL) and one soluble (HoxEFUYH) [NiFe] hydrogenases in *Thiocapsa roseopersicina* BBS, a purple sulfur photosynthetic bacterium. The majority of genes coding for accessory proteins that participate in the biosynthesis and maturation of hydrogenases seem not to be located in the vicinity of the hydrogenase structural genes. Transposon-based mutagenesis was used to locate the hydrogenase accessory genes. Molecular analysis of strains showing mutant phenotypes led to the identification of *hupK* (*hoxV*), *hypC1*, *hypC2*, *hypD*, *hypE*, and *hynD* genes. The roles of *hynD*, *hupK* and the two *hypC-s* genes were investigated in detail [1].
The putative HynD was found to be a hydrogenase specific endoprotease type protein, participating in the maturation of the HynSL enzyme. HupK plays an important role in the formation of the functionally active membrane-bound [NiFe] hydrogenases, but not in the biosynthesis of the soluble enzyme. In-frame deletion mutagenesis showed that HypC proteins were not specific for the maturation of either hydrogenase enzyme. The lack of either HypC proteins drastically reduced the activity of every hydrogenase. Hence both HypC-s might participate in the maturation of each [NiFe] hydrogenases. Homologous complementation with the appropriate genes substantiated the physiological roles of the corresponding gene products in the H_2 metabolism of *T. roseopersicina*.

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P3-4. Assembly of a Functional Cyanobacterial Hydrogenase

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Enzymes directly involved in hydrogen metabolism in cyanobacteria are [NiFe]-hydrogenases and nitrogenases. The genes encoding the structural part of the uptake hydrogense in cyanobacteria are *hupSL* respectively *hoxEFUYH* for the bi-directional hydrogenase. To obtain an active hydrogenase several maturation proteins, the *hyp*-genes, are also required [1]. The *hyp*-genes are believed to take part in the insertion of iron and nickel in the active site as well as the incorporation of the CN- and CO-ligands into the iron atom [2]. In our project we focus on enzymes involved in the assembly of functional hydrogenases. Little is known about the function of cyanobacterial *hyp*-genes. However, through similarity studies with e.g. *Escherichia coli* a number of genes have been recognized as homologues with genes of known functions [3]. We have so far identified seven putative maturation proteins in Nostoc ATCC 29133; *hypCDEFAB* [4] and *hupW* [5]. *hypCDEFAB* are transcribed as one unit and are located approximately 3.8 kb upstream, but in opposite direction of *hupSL*. The gene products of *hupW* of *Nostoc* ATCC 29133 (uptake hydrogenase only), and *hupW* and *hoxW* of *Nostoc* PCC 7120 (both uptake and bi-directional hydrogenases), are proteases involved in the proteolytic cleavage of the large subunit of the respective hydrogenase. This cleavage induces a conformal change, positioning the active centre correctly in the enzyme [6]. Recent data will be presented and discussed.

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P3-5. Specific accessory proteins involved in maturation of the membrane-bound [NiFe] hydrogenase in *Ralstonia eutropha* H16

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H_2-dependent lithoautotrophic growth of the beta-proteobacterium *Ralstonia eutropha* H16 is based on two energy-generating [NiFe] hydrogenases: a soluble, cytoplasmic, NAD-reducing hydrogenase and a periplasmically oriented, membrane-bound hydrogenase (MBH) [1]. The MBH consists of a large subunit harbouring the catalytically active [NiFe] site and a small subunit containing [Fe-S] clusters that conduct the electrons to a membrane integral cytochrome b. This component is bifunctional since it anchors the hydrogenase dimer to the cytoplasmic membrane and links the MBH to the respiratory chain [2]. The MBH undergoes a multistep maturation pathway including: incorporation of the metal center into the large subunit, endoproteolytic processing, oligomerization of the subunits, and Tat-mediated membrane translocation [3]. Eght MBH-specific accessory genes are involved in the synthesis of active MBH [4]. In this study, we analysed isogenic deletion mutants in the accessory genes *hoxL*, *hoxO*, *hoxQ*, *hoxR*, *hoxT* and *hoxV*. We present evidence that the gene products of *hoxO* and *hoxQ*, both indispensable for MBH maturation, directly interact with the precursor of the MBH small subunit that contains a Tat-signal peptide. Based on characterisation of the mutants, possible functions of the MBH accessory proteins will be discussed.

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P3-6. Characterization of the hydrogenase accessory genes in *Synechocystis* sp. PCC 6803

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The seven accessory genes *(hyp A, B, C, D, E, F* [1] and the protease *hoxW)* for the maturation of the NiFe-hydrogenase are scattered over the whole genome of *Synechocystis* sp. PCC 6803. In contrast the two homologues for *hypA* and *hypB* are encoded in the same gene-cluster. Deletion mutants were produced to test the effect of the deleted genes on hydrogenase activity. All mutants completely lack hydrogenase activity except □*hypA2* and □hypB1. These two mutants almost show wild-type hydrogenase activity. The *hypA*- and *hypB*-genes are involved in Ni-insertion into the large subunit as shown for other organisms [2, 3]. As the deletion of *hypA2* and *hypB1* (proposed function in Ni-metabolism) have no influence on hydrogenase activity, the effect on urease as another Ni-containing enzyme was tested. The two proteins seem to have no effect on urease activity. It seems as if there is another Ni-enzyme and a good candidate has to be tested.

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P3-7. Coordinating assembly and export of complex bacterial proteins

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In *Escherichia coli* a subset of periplasmic proteins are synthesised with specialised N-terminal signal peptides containing a distinctive SRRXFLK 'twin-arginine' amino acid sequence motif. Precursor proteins bearing twin-arginine signal peptides are targeted post-translationally to the twin-arginine translocation (Tat) system. The majority of proteins targeted to the Tat pathway in *E. coli* contain redox-active cofactors that must be inserted prior to export of the fully folded proteins. It is likely that cellular mechanisms exist that prevent either wasteful export of immature substrates or competition between immature and mature proteins for the transporter. In this work, this 'proofreading' activity has been investigated using trimethylamine N-oxide reductase (TorA) and the membrane-bound [NiFe] hydrogenases as model systems. The accessory protein TorD interacts with TorA during loading of a molybdenum cofactor prior to the transport event. Here, we show by a combination of genetic and biochemical approaches that TorD recognises at least two independent binding-sites on the TorA precursor - one of which is the twin-arginine signal peptide itself. We propose TorD operates as a proofreading chaperone preventing premature targeting of immature TorA during maturation. In order to identify other system-specific chaperones involved in targeting or proofreading Tat substrate assembly, experiments were designed using a bacterial two-hybrid system. A chaperone network was established for [NiFe] Hydrogenases-1 - and -2 where the HyaE accessory protein was shown to interact with the Tat signal peptide bearing HyaA subunit of hydrogenase-1 and HybE with the hydrogenase-2 Tat signal bearing subunit HybO. A *hybE* knockout resulted in almost complete loss of hydrogenase-2 activity due to uncoordinated assembly and export processes.

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P3-8. Heterologous production of active [NiFe] hydrogenases

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From a basic research perspective, development of recombinant (over)production systems for catalytically active [NiFe] hydrogenases in organisms which do not possess any hydrogenase activity is required to establish the minimal genetic information neccessary for synthesis and maturation of these complex enzymes. Such overproduction systems will also prove valuable in biotechnological applications since acquisition of an uptake hydrogenase enables an organism to use the simple molecule H_2 as alternative and relatively cheap energy source. Here we show the heterologous production of two physiologically distinct [NiFe] hydrogenases of the beta proteobacterium *Ralstonia eutropha*: (i) The membrane-bound hydrogenase, the *R. eutropha* hydrogenase with the most complex maturation pathway, and (ii) the cytoplasmic regulatory hydrogenase (RH) which serves as simpler model. Both proteins were actively produced in the gamma proteobacteria *Pseudomonas stutzeri* and *Escherichia coli*. Furthermore, the heterologously produced RH was purified from *E. coli*, and spectroscopic analysis revealed that the structure of the active site is indistinguishable from that of the homologously produced enzyme. The requirements as well as the constraints for the production of both enzymes in their heterologous hosts will be discussed.

P4-1. Sequencing of *Desulfovibrio vulgaris* Miyazaki F [NiFe] hydrogenase operon and hydrogenase knock-out mutant generation

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The structural genes *hynA* and *hynB* coding for the small and large subunit of [NiFe] hydrogenase, were known for *Desulfovibrio vulgaris* Miyazaki F [1]. The sequence homology from related species, known to have genes for structural and maturation subunits arranged in the big operons was used to confirm the presence of *hyn C* in DvMF. Selecting the correct downstream sequence of the known chromosomal DNA region using the known region as probe for Southern blot, the complete sequence of the operon consisting of *hynA*, *hynB*, *hynC* and *hynD* was established.
Due to their complex biosynthesis, hydrogenases can currently only be expressed in their genuine host. A mutation analysis thus requires the deletion (knock-out) of the wild-type gene and the subsequent expression of a mutant protein. A deletion mutant was generated by using a suicide plasmid. The plasmid contained the hydrogenase gene operon having part of *hynA*, all of *hynB* and part of *hynC* deleted and replaced by the chloramphenicol resistance gene. Homologous recombination of the native operon with the cloned one resulted in knock-out of the gene. This was confirmed by using PCR, Southern, and Western blots.

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P4-2. An FNR-type regulator controls the anaerobic expression of Hyn hydrogenase in *Thiocapsa roseopersicina*

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The purple sulfur photosynthetic bacterium, *Thiocapsa roseopersicina* BBS, contains a heat-stable membrane-associated hydrogenase coded by the *hyn* operon. Expression from the *hyn* operon regulatory region is up-regulated under anaerobic conditions. Activating *cis* elements were mapped between positions -602 and -514 upstream from the start site of the *hynS* gene. Within this region two sequences resembling the FNR binding sites were recognized. The gene of an FNR homologue, FnrT, was identified in the genome of *T. roseopersicina* and an *fnrT* knockout mutant was constructed. The anaerobic induction of *hyn* expression is abolished in the *fnrT* mutant suggesting that FnrT is an activator of the *hyn* promoter. The *T. roseopersicina hyn* promoter can be activated in *Esherichia coli* and *Rhodobacter capsulatus*, as well. The anaerobic regulation is dependent on FNR both in *E. coli* and *R. capsulatus*. *In vitro* experiments using purified *E. coli* Ala154 FNR protein and purified *E. coli* RNA polymerase show that two sites are occupied by FNR activating transcription initiation at the *hyn* promoter, and that FNR binding at the two target sites activates initiation by different extents.

P4-3. Role and regulation of the bidirectional hydrogenase of *Synechocystis* sp. PCC 6803

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The bidirectional hydrogenase of *Synechocystis* was shown to work as an electron valve under rapidly changing environmental conditions like dark-light and light intensity shifts [1]. Investigation of different deletion mutants of important electron transport components were made to get insights into the regulation of the hydrogenase. Mutants missing terminal oxidases showed a considerably higher hydrogenase activity compared to wild type cells. These findings underline the function of the hydrogenase as a valve for low potential electrons. In addition they confirm that the redox conditions of the thylakoid membrane control the expression of this enzyme [2]. It was found that the photohydrogen production is increased under conditions when the NDH-M complex is repressed. Since the NDH-M complex was shown to be part of the cyclic electron transport [3], the recycling of electrons plays an important role in redox poising under rapidly changing conditions. Mutants with an impaired expression of the NDH-L complex showed an additional increase of photohydrogen production. This supports that in addition to cyclic electron transport the oxidation of NADPH by this complex is also a possibility to control the redox state of the cell. Our results support that the so called bi-directional hydrogenase of *Synechocystis* eliminates an excess of reducing equivalents by producing hydrogen when the conditions get highly reducing. It still remains to be shown if this enzyme is part of the NDH complex making up the NADPH-binding subunits [4] or if it is a separate unit.

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P4-4. Effect of deletion mutations of electron transport components on the hydrogen metabolism and regulation of the hydrogenase of Synechocystis sp. PCC 6803

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Cyanobacteria are unique, since their thylakoid membranes contain all the electron transport complexes necessary for a photosynthetic as well as respiratory electron transport. Three different types of respiratory terminal oxidases were characterized in the cyanobacterium *Synechocystis* PCC 6803 [1,2]. One of them is directly linked to the plastoquinone pool and is working as a quinol-oxidase [3]. The other two seem to be cytochrome c oxidases. The complex I or NADPH:plastoquinone oxidoreductase (Ndh) can be separated in at least two different types, containing different copies of the NdhD subunit [4]. In addition there are two different types of succinate-deydrogenase (Sdh) [5]. The effect of deletion of *ndh*- and *sdh*-genes and different sets of terminal oxidases on hydrogenase expression and hydrogen metabolism was investigated. Deletion of *ndhD1* results in a considerable hydrogen production at the onset of light. In contrast to this all other *ndhD* single mutants produced no hydrogen. Deletion of at least two of the oxidases results in photohydrogen production at the onset of light. Additionally the expression level of the hydrogenase was found to be higher in different oxidase mutants. Deletion of genes of the succinate-dehydrogenase had no effect on hydrogenase expression or hydrogen evolution. New results about the regulation of the hydrogenase operon and a model concerning the interaction of the hydrogenase with the electron transport will be presented. The effect of the redox state of different electron transport components on the expression of the hydrogenase will be discussed.

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P4-5. On the physiological role of the Hox and membrane-bound hydrogenases in *T. roseopersicina*

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The phototrophic purple sulfur bacterium *Thiocapsa roseopersicina* contains two membrane associated [NiFe] hydrogenases (HupSL and HynSL) and one cytoplasmic cyanobacterial-type, heteropentameric HoxEFUYH hydrogenase [1]. The organization of the *hyn* operon suggests, that it codes for a heterotetrameric enzyme, while the heterotrimeric HupSLC enzyme resemble the uptake hydrogenases of other microbes, which recycle the hydrogen produced by the nitrogenase. To establish the real physiological functions of the hydrogenases, their structural genes have been deleted in all combinations, and the mutant cells containing one of these enzymes were investigated. The absence of the structural genes coding for both membrane-bound hydrogenases did not abolish HypF dependent hydrogen evolving capacity of the cells under non-nitrogen-fixing conditions. This hydrogen-evolving capacitiy was dependent on the light/dark shift and the amount of the sulfur compound added to the medium. At high thiosulfate concentration the hydrogen evolution was light dependent, while decreasing the amount of thiosulfate resulted in diminished hydrogen evolution under light, but the H_2 production could be induced in dark. In frame deletion of the *hoxE* gene abolished the hydrogen evolution derived from Hox enzyme *in vivo*, although had no deleterious effect on the NAD^+-reducing hydrogenase activity in vitro suggesting, that HoxE has a hydrogenase related role, likely in the electron transfer processes. *T. roseopesicina* could utilize hydrogen for its growth in the presence of tiny amount of sulfide. A systematic study addressing the question of which enzyme is required for the hydrogen utilization might provide a deeper insight into the actual physiological role of these structurally diverse isoenzymes.

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P4-6. Increased hydrogen production by E*scherichia coli* strain HD701 from in comparison with the wild type parent strain MC4100

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Hydrogen production by *E. coli* is mediated by the formate hydrogenlyase (FHL) complex. *E. coli* strain HD701 cannot synthesize the FHL complex repressor, Hyc A. Consequently, it has an up-regulated FHL system and can therefore evolve hydrogen at a greater rate than its parental wild type, *E. coli* MC4100. Results have shown that over range of glucose from 3 mM to 200 mM strain HD701 evolved several times more hydrogen than MC4100. Studies using industrial wastes of a high sugar content yielded similar results demonstrating the industrial potential of strain HD701 for commercial hydrogen production. Optimisation tests on strain HD701 determined that 100 mM is the optimum concentration of glucose for hydrogen production. Any concentration higher than 100 mM did not increase the yield of hydrogen. Increasing the temperature from room temperature (18-20°C) to 30 °C increased the rate of hydrogen production by strain HD 701 by a factor of *ca.* 4. However, decreasing the initial pH from 7.3 to 5.5 significantly decreased hydrogen production. A 50% (v/v) inoculum was found to be the optimum; increasing the inoculum above 50% did not increase hydrogen production, indicating that this is the cell concentration at which substrate availability becomes limiting. Stirring the medium doubled the rate of evolution of hydrogen. Combining these optimised conditions was found to increase the rate of hydrogen production by *ca.* 6 times compared to initial conditions, from 5.2 ± 0.77 ml h^{-1} L^{-1} to 29.8 ± 0.81 ml h^{-1} L^{-1}. Scaling up the system to a 5L reactor produced the evolution rates from 100 mM glucose and caramel of *ca.* 55 ml h^{-1} L^{-1}.

P4-7. Molecular characterization of uptake hydrogenase in *Frankia*

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Hydrogenases are microbial enzymes that catalyze the reversible oxidation of molecular hydrogen. From the previous physiological studies it is evident that uptake hydrogenases are common among *Frankia* strains. Here, we *present characterization at a molecular level of uptake hydrogenase in Frankia* using immunology, mass spectrometery,PCR and sequence analysis. Antibodies raised against the large subunit of Ni-Fe hydrogenase of *Ralstonia eutropha* (HoxG) recognised a polypeptide at about 60 kDa, corresponding to the large hydrogenase subunit in *Frankia* UGL011102. Spots identified by 2-D immunoblots in replicate gels were picked at app. 60 kDa, digested and analysed using MALDI Q-tof. The peptide sequence analysis revealed two Frankia peptide sequences VQGVGFRP and LNLSGDEA. Blast searches of the NCBI Protein database for short nearly exact matches showed a similarity of 100 % with a hydrogenase maturation protein HYPF of *Rhodobacter capsulatus* (accession no. Q02987) and a similarity of 75% with a hydrogenase expression/maturation protein HYPE of *Azotobacter vinelandii* (accession no. P40595), respectively. Furthermore, a 549 bp long PCR-product with 77% DNA sequence similarity to small subunit of *Bradyrhizobium japonicum* was used in a successful attempt to sequence the uptake hydrogenase of Frankia sp. ACN14a, and the organization of structural *hup* genes in Frankia was revealed. By use of a Genoscope Frankia genome sequence, a 700 base pair long DNA sequence with 75% similarity to *R. eutropha hoxG* encoding the large subunit of uptake hydrogenase could also be shown in 4 *Frankia* strains using PCR. This study hints a close relation between *Frankia* hydrogenase and hydrogenases from other organisms.

P4-8. Hydrogen metabolism in a filamentous non-heterocystous N2-fixing cyanobacterium isolated from the North Sea: *Lyngbya aestuarii* CCY 9616

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Lyngbya aestuarii CCY 9616 is a marine filamentous non-heterocystous cyanobacterium isolated from the intertidal flats of the island Mellum in the North Sea [1]. Although being a nitrogen-fixing strain, preliminary studies indicated that in contrast with all N_2-fixing cyanobacteria studied so far [2], the uptake hydrogenase could be absent in *L. aestuarii* [3]. In order to evaluate the presence/absence of the genes encoding a nitrogenase (*nif*), an uptake hydrogenase (*hup*), and a bidirectional hydrogenase (*hox*), oligonucleotide primers designed against conserved regions within other cyanobacteria were used. The presence of those genes was confirmed by sequencing 1039 bp within *nifK*, 1419 bp within *hupL*, and 282 bp within *hoxH*. To establish if the three genes were expressed, RT-PCR studies were performed. Preliminary results indicate that *nifK* is expressed both in light and dark in *L. aestuarii* grown under N2-fixing-conditions and alternating 16h light / 8h dark cycles, showing higher expression from the end of the light period until the end of the dark phase. The nitrogenase activity, measured by the on-line acetylene reduction assay, reaches a clear maximum within the dark period. In agreement, with the transcript level of nifK, the hup*L* expression was also higher in the end of the light phase and in the beginning of the dark phase. A very low expression of hox*H* was detected in both N2-fixing and non-N2-fixing conditions.
Further studies are required to elucidate the correlation between the physiological conditions and the activity of the three enzymes in *L. aestuarii*.

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P4-9. *hupSLW* in *Gloeothece* sp. ATCC 27152: the first molecular data on an uptake hydrogenase from a unicellular N_2-fixing cyanobacterium

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Up to now, only limited amounts of biochemical/physiological data are available concerning uptake hydrogenases in unicellular cyanobacteria [1, 2, 3]. Recently, in the unicellular N_2-fixing cyanobacterium *Gloeothece* sp. strain ATCC 27152, the unequivocal presence of an uptake hydrogenase was reported. In contrast, no hybridization signals could be detected when probes for *hox* genes were used [3]. Here we present the first comprehensive molecular data on an uptake hydrogenase being present in a unicellular cyanobacterium. The structural genes (*hupSL*) encoding an uptake hydrogenase in *Gloeothece* sp. ATCC 27152, a strain capable of aerobic N2-fixation, were identified and sequenced. 3´-RACE experiments uncovered the presence of an additional ORF 184 bp downstream of *hupL*, showing a high degree of sequence identity with a gene encoding an uptake hydrogenase specific endopeptidase (*hupW*) in other cyanobacteria. In addition, the transcription start point was identified 238 bp upstream the *hupS* translational start. RT-PCR experiments revealed that *hupW* is co-transcribed with the uptake hydrogenase structural genes in *Gloeothece* sp. ATCC 27152. A putative NtcA binding site was identified in the promoter region upstream of *hupS*, centered at -41.5 bp with respect to the transcription start point. Electrophoretic retardation of a labeled DNA fragment (harboring the putative NtcA binding motif) was significantly effected by an *Escherichia coli* cell-free extract containing over-expressed NtcA, suggesting that NtcA is involved in the transcriptional regulation of *hupSLW*. Transcriptional studies using Northern hybridizations are being performed, and preliminary data will be presented.

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P4-10. Effect of Nickel (Ni) and Iron (Fe) concentrations on expression level of uptake hydrogenase genes of *Rhodobacter sphaeroides* O.U.001

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In photosynthetic non-sulfur bacteria, hydrogen production is catalysed by nitrogenases and hydrogenases. Hydrogenases are metalloenzymes that are basically classified as Fe hydrogenases, Ni-Fe hydrogenases and metal-free hydrogenases. Two distinct Ni-Fe hydrogenases are described as uptake hydrogenases and bidirectional hydrogenases. The uptake hydrogenases are membrane bound dimeric enzymes consisting of small (*hupS*) and large (*hupL*) subunits, and are involved in uptake and recycling of hydrogen providing energy for nitrogen fixation and other metabolic processes. In this study, the presence of uptake hydrogenase genes was shown in *Rhodobacter sphaeroides* O.U.001 strain and the part of uptake hydrogenase *hupS* gene sequence was determined for the first time. The sequence showed 93% of similarity with the uptake hydrogenase *hupS* of *R. sphaeroides* R.V. There was no significant change in growth of the bacteria at different concentrations of metal ions namely nickel, molybdenum and iron. The effect of metal ions on hydrogen production was also studied. The maximum hydrogen gas production was achieved in 8.4 μM of nickel and 0.1 mM of iron containing media. The transcriptional regulation of uptake hydrogenase genes were examined by RT-PCR. Increasing the concentration of nickel up to 8.4 μM increased the expression of *hupS*. At varied concentrations of Fe-citrate (0.01 mM - 0.1 mM) expression of *hupS* was not detected until hydrogen production stopped. These results indicated that the expression of uptake hydrogenase genes were dependent on nickel and iron concentrations at different phases of growth. The results will be significant for the improvement strategies of *R. sphaeroides* O.U.001 to increase hydrogen production efficiency.

P4-11. Hydrogen and Sulphur Metabolism in the Hyperthermophilic Bacterium *Aquifex aeolicus*

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The most hyperthermophilic bacteria known to date are members of the genus *Aquifex*. This organism, which is microaerophilic, hydrogen-oxidizing, obligate chemolithoautotroph, represents the earliest branching order in the bacterial domain. The genome of *Aquifex aeolicus* has been completely sequenced. Three [NiFe] hydrogenases are annotated in this genome. Two are membrane-bound in the periplasm (hydrogenases I and II) and one is soluble in the cytoplasm (hydrogenase III). Two of them (hydrogenases I and III) have been purified in the group and a model for the metabolic roles of the three enzymes has been proposed [1].
A. aeolicus is usually cultured at 85°C under $H_2/CO_2/O_2$ atmosphere in a medium containing only inorganic compounds. By comparison with a close relative, *Aquifex pyrophilus* which can use elemental sulphur as electron acceptor, we have replaced thiosulphate by sulphur in the medium and shown that *A. aeolicus* can use hydrogen as electron donor and S^0 as electron acceptor. A membrane-bound sulphur-reducing complex transferring electrons from H_2 to S^0 has been purified. Such multi-proteins complexes have been purified from only two other hyperthermophilic organisms [2,3]. Twelve proteins have been identified by mass spectrometry and the majority are known to be involved in electron transport chain or energy conservation. Hydrogenases I and II are present in this macromolecular complex with a membrane-bound sulphur-reductase containing molybdenum and the bc1 complex. This complex has been characterised with respect to biochemical/biophysical parameters, activity and substrates specificity. A model for hydrogen/sulphur respiration in *Aquifex aeolicus* has been proposed.

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P4-12. Establishment of a *Rhodobacter capsulatus* expression system for complex enzymes: uptake hydrogenase as an example

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In many cases enzymes of biotechnological interest consist of more than one subunit or harbouring a complex cofactor. Synthesis of such biocatalysts requires an expression system which allows the joint transcription of a larger number of genes which are under control of a strong and inducible promoter. In the presented work we chose the uptake hydrogenase of the photosynthetic non-sulfur purple bacterium *Rhodobacter capsulatus* as an example for such complex enzymes. The uptake hydrogenase consistS of two subunits (HupS and HupL) and carries the Ni-Fe center as an active site. Synthesis of active hydrogenase needs the expression of at least 17 genes, whose products are involved in maturation of uptake hydrogenase. All genes are organized in one cluster and are oriented in the same direction. We have started to generate a high- level expression system which combines a T7 polymerase based system with different, strong and tightly controlled *R.capsulatus* promoters. This expression system consists of two components: I. T7 RNA polymerase gene which is integrated into the *R. capsulatus* chromosome (e.g. in the *recA* gene) or is lying onto a broad host range vector. In both cases, expression of T7 polymerase gene is under control of either *Escherichia coli lac* promoter or *R. capsulatus fru* promoter. II. Plasmid based or integrated T7 promoter which is lying upstream of gene(s) of interest. The new system will allow us to overexpress the wild-type hydrogenase as well as molecular engineered hydrogenases which are more favorable for biotechnological applications.

P4-13. Effect of Genetically Modified Electron Carrier Cytochromes on Hydrogen Production in *Rhodobacter capsulatus*

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The facultative photosynthetic bacterium *Rhodobacter capsulatus* grows under various environmental conditions by using different energy generating processes including photosynthesis, respiration, and hydrogen oxidation. During these energy conversion metabolisms, electrons are transferred to hydroquinone pool through dehydrogenases, photosynthetic reaction center, and membrane bound uptake hydrogenases. *R. capsulatus* has two c-type electron carrier cytochromes (cyt); the soluble cyt c2 and the membrane-attached cyt cy, that act as electron carriers during photosynthetic and respiratory growth of this species (1). Previously, soluble form of cyt cy was constructed by fusing genetically the signal sequence of cyt c2 to the cyt c domain of cyt cy. The obtained novel soluble cyt cy was unable to support photosynthetic (Ps) growth of a *R. capsulatus* strain that lacks both the cyt c2 and cyt cy but yielded Ps^+ revertants frequently. Photosynthetic electron transfer properties of some of these revertants were analyzed and compared with the cyt cy and cyt c2 by light-activated time-resolved spectroscopic measurements. In this study, various *R. capsulatus* strains harbouring genetically modified electron carrier cyts were compared with several wild type *Rhodobacter* species to determine the effect of these genetically modified electron carrier cyts on the generation of the reducing equivalents and the hydrogen production metabolism (2). Further analysis of the hydrogen metabolism by modification of the hydroquinone oxidase dependent alternate respiratory pathway is also being pursued.

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P5-1. Utilization of Different Organic Acids by *Rhodobacter sphaeroides* O.U. 001 for Biological Hydrogen Production

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Rhodobacter sphaeroides O.U. 001 is a purple non-sulfur bacteria with the nitrogenase-hydrogenase enzyme system. Under anaerobic conditions and illumination, it evolves H_2 from breakdown of organic acids. Growth and H_2 production of *Rhodobacter sphaeroides* O.U. 001 was investigated in media containing six different carbon sources; malate (15mM), acetate (30mM), propionate (20mM), lactate (20mM), butyrate (15mM) and pyruvate (20mM). Na-glutamate was used as nitrogen source. Carbon/nitrogen ratio was 15/2. The inoculum was grown in malate containing medium. All of the experiments were carried out in 55 ml gas-tight glass bioreactors. The results show that *Rhodobacter sphaeroides* O.U. 001 was capable to metabolize all of these different carbon sources tested, however the growth and hydrogen production varies. The amounts of produced H_2 were 1.0, 1.0, 1.0, 0.9, 0.8, 0.5 ml/ml culture for malate, propionate, pyruvate, acetate, lactate and butyrate media respectively. Malate was the best carbon source for H_2 production, this result was in accordance with the previous literature [1]. The highest hydrogen amount, yield, substrate conversion efficiency, light conversion efficiency and rate were obtained with malate. The drawback of the malate was its availability and high cost for large scale hydrogen production compared to others. Highest biomass accumulation was observed in acetate medium. However if the inoculum was grown in acetate medium instead of malate medium, only trace amount of hydrogen production was observed. Pyruvate, lactate and propionate were also efficiently used by the bacteria for growth and H_2 production. However butyrate was the least efficient carbon source among all. Possibly this was due to the ease of conversion of butyrate to poly-ß-hydroxybutyrate [2].

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P5-2. Photobiological Hydrogen Production from Different Olive Mill Wastewater Samples

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Organic wastes of various sources with low nitrogen contents are the most promising substrates for hydrogen photoproduction with the advantage of lower costs. Photobiological hydrogen production from different olive mill wastewater (OMW) samples was investigated by *Rhodobacter sphaeroides* O.U.001 at 55 mL penicillin bottles under anaerobic conditions at 32°C. The illumination was provided by a 150 W tungsten lamp. Hydrogen production media include 4% (v/v) of OMW in distilled water. *Rhodobacter sphaeroides* is a purple non-sulfur bacterium with the nitrogenase-hydrogenase enzyme system. The physical and the chemical composition of OMW samples depend on local and seasonal factors such as the processed olive; harvesting time; cultivation area; and oil extraction technique. OMW samples contain significant amounts of chemical (COD = 30 -75 g/L) and biological oxygen demand (BOD = 15-30 g/L) values, which indicate the high amount of organic matter content [1]. Hydrogen production yields of these OMW samples were between 5 - 18 L_{H2} / L_{OMW}. The highest hydrogen production (18 L_{H2} / L_{OMW}) was observed for the sample which has the lowest phenol (0.06 g/L) and total solid (17.75 g/L) content in accordance with its brighter color. It also has the highest carbon to nitrogen molar ratio (73.8) that is known to enhance the photosynthetic efficiency of the medium. On elemental basis it has the highest Mo (1.78 ppm) and the lowest Ni (0.1 ppm) content since restrictions of nickel element is known to decrease the synthesis of the uptake hydrogenase enzyme that utilizes the hydrogen gas. This result also supports the literature that the inhibition of uptake hydrogenase is a prerequisite for an efficient photoheterotrophic H_2 production [2].

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P5-3. Using biohydrogen evolved by *Escherichia coli* HD701 as an electron donor for the bio-reduction of Pd (II) to Pd (0)

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An *E. coli* strain HD701, which cannot synthesise the FHL repressor (Hyc A), and is therefore up-regulated with respect to FHL expression, has been constructed . Previous work by Lloyd *et al* with this strain showed that its up-regulated FHL expression allowed it to reduce technetium (via the hydrogenase 3 component acting catabolically at the expense of formate or hydrogen) at a higher rate compared to its parental wild type *E. coli* MC4100. Investigating the hydrogen production by strain HD701showed that evolved significantly more hydrogen than strain MC4100. Moreover the study demonstrated the potential viability for using the process on an industrial scale; it was shown that this strain can utilise industrial sugar wastes, for hydrogen production. The process has been optimised and simplified for high efficiency with respect to hydrogen production and its operating procedure. Results obtained from a 6 L batch reactor using industrial confectionery wastes gave rates of between. 1.5 L and 2 L/day/L culture. The majority of work to date has only concentrated on optimisation of the hydrogen production processes and not the potential applications of the evolved gas. This study was aimed also at investigating the use of the biohydrogen generated by *E. coli* HD701 in the reduction of Pd (II) to Pd(0), (a widely used industrial catalyst). Reduction of palladium was carried out using a synthetic 100 mM glucose solution and also a confectionery waste as an oxidisable carbon source for generation of the biohydrogen by the *E. coli*. The evolved biohydrogen was then directed into the palladium reduction column which contained a suspension of *Desulfovibrio desulfuricans* resting cells preincubated with 2 mM tetrachloropalladate solution or one of two palladium-containing industrial wastes with. In all cases, Pd (II) was completely reduced to biomass bound Pd(0) by the biohydrogen. The time taken for this to occur was comparable to that obtained using commercially available hydrogen.

P5-4. Biological Reduction of Cr(VI) using biohydrogen evolved by *Escherichia coli* strain HD701 from sugar

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The potential to use biologically produced hydrogen as an electron donor for Cr(VI) reduction by *Desulfovibrio vulgaris* NCIMB 8303 and palladised bacteria (Bio-Pd°) was investigated. Biological hydrogen was produced under anaerobic conditions by *Escherichia coli* HD701(a strain up regulated with respect to formate hydrogenlyase expression) using glucose, nougat or caramel wastes as a carbon source. Evolved hydrogen was fed into a batch Cr(VI) reducing reactor containing either free or immobilized *D. vulgaris* or Bio-Pd°, and the rate of Cr(VI) reduction was monitored. Results show that biohydrogen can be used as an electron donor for Cr(VI) reduction by free and agar-immobilized *D. vulgaris* and Bio-Pd°. Further studies investigating the potential of bio-hydrogen as an electron donor for continuous Cr(VI) reduction by agar-immobilized Bio-Pd° showed that 100 % reduction efficiency could be obtained.

P5.5. Hydrogenase assisted reduction of Palladium by mutants of *Desulfovibrio fructosovorans*

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To elucidate the role of different hydrogenases in palladium reduction, palladised biomass (Bio-Pd) of wild type *D. fructosovorans* and three mutants with deactivated soluble periplasmic hydrogenases ([Fe]-only, [Ni-Fe]- and [Fe]-[Ni-Fe] double hydrogenase mutants) were prepared and studied via electron microscopy (TEM). Mutants were constructed using marker exchange mutagenesis [1] and were challenged with soluble Pd(II). In the initial stage of Pd (II) reduction within the wild type cells, Pd nanoparticles were seen distributed throughout the periplasm. However, within double mutant cells the Pd crystal growth (5-10 nm) was observed only on the surface of the inner membrane, which is the site of localisation of the remaining membrane-bound hydrogenase. This suggests the role of hydrogenase in the reduction of Pd(II) to Pd(0). In order to verify this observation, wild type cells containing a full complement of hydrogenases were treated in a way such that the integrity of the cells was maintained and all soluble proteins, including soluble [Fe]-only and [Ni-Fe] hydrogenases, were extracted. The obtained cells were biochemical analogues to genetically prepared double mutant cells in respect to hydrogenase activity distribution within periplasm. These 'periplasmically treated' cells were challenged with Pd(II) solution and reduction of Pd was observed. The TEM showed the Pd-nanoclusters formation on the inner cell membrane, similar to the pattern of Pd nanoparticles within the genetically obtained double hydrogenase mutant cells. We propose that in the absence of soluble hydrogenases the reduction of Pd occurs on the hydrogenase-containing inner periplasmic membrane, causing the appearance of small Pd nanoparticles.

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P5-6. Magnetic properties of Palladium nanoparticles in hydrogenase negative mutants of *Desulfovibrio fructosovorans*

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